Developing a rapid, sensitive and accurate Malaria detection scheme using Reverse Transcription Loop Mediated Isothermal Amplification coupled CRISPR-Cas module

A Thesis

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by

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From January 2023 to December 2023

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled 'Developing a rapid, sensitive and accurate Malaria detection scheme using Reverse Transcription Loop Mediated Isothermal Amplification coupled CRISPR-Cas module' 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 Nirav Saharey at Indian Institute of Science Education and Research under the supervision of Dr. Krishanpal Karmodiya, Associate Professor, Department of Biology, during the academic year 2022-2023.

Dr. Krishanpal Karmodiya

Committee:

Name of your Guide - Dr. Krishanpal Karmodiya

Name of your TAC - Dr. Siddhesh S. Kamat

This thesis is dedicated to

To my family

Declaration

I hereby declare that the matter embodied in the report entitled Developing a rapid, sensitive and accurate Malaria detection scheme using Reverse Transcription Loop Mediated Isothermal Amplification coupled CRISPR-Cas module are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Krishanpal Karmodiya and the same has not been submitted elsewhere for any other degree

Nirav

Your Name: Nirav Saharey

Date: 20th October 2023

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Abstract

Malaria remains a public challenge infecting millions and killing thousands of people even today. The major hurdle in the eradication of this infection is the late diagnosis and persistence of asymptomatic carrier cases in the population. The diagnostic technologies available for detection are either insensitive for early detection (RDTs) or expensive in terms of time, money, labour and resources needed (PCR, microscopy). Even for symptomatic illnesses, their accuracy is restricted, and since asymptomatic patients have low parasite burdens, it is even difficult to detect them. Therefore, we need a low-cost diagnostic tool that can identify infections in their early stages and can be used in resource-constrained settings without the aid of trained staff. The equipment must be labour-saving for mass diagnosis and provide a quick, straightforward readout for simple interpretation. We are developing a low-resource, user-friendly diagnostic kit incorporating the Reverse Transcription Loop-Mediated Isothermal Amplification technique of nucleic acid amplification coupled with the CRISPR Cas system for parasite detection in blood samples. Using this technology we have shown detection at sub-microscopic levels. We were able to detect parasites at 0.000002% parasitemia, this implies our detection tool is sensitive enough for detection at the levels of 1 to 2 parasites in 10 microliters of blood. Achieving sensitivity higher than the parasitemia ranges present in asymptomatic cases. Developing this module as a field deployable diagnostic kit will have a great impact on malaria eradication efforts. With this kind of module early detection and screening of asymptomatic cases is possible which eventually will lead to, disrupting the chain of infection. Since the module is designed for operability under resource-constrained settings, with minimal training, hence simplifies diagnosis even in remote rural places.

Acknowledgments

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I am grateful to IISER Pune for hosting me and providing this platform to carryout the research. It was an exciting journey throughout.

To my friends on campus, you have been the vibrant backdrop to my college years. Your friendship, laughter, and the countless shared experiences have made these years truly unforgettable. You have been a source of not only inspiration but also unwavering support, filling my college days with meaning.

To my family, I owe profound appreciation for their unwavering love, encouragement, and patience throughout this demanding process. Their steadfast support has been a constant source of strength.

This thesis symbolizes a pursuit of knowledge and a contribution to the advancement of our understanding in malaria diagnostics. It has been an enriching voyage, made possible by the collective efforts of those who have contributed, in ways both small and substantial.

Contributions

Contributor name	Contributor role
KK, NS	Conceptualization Ideas
NS	Methodology
KK, RM	Training
—	Software
NS	Validation
NS	Formal analysis
NS	Investigation
KK, NS	Resources
NS	Data Curation
NS	Writing - original draft preparation
KK, NS	Writing - review and editing
—	Visualization
KK	Supervision
KK	Project administration
KK	Funding acquisition

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(KK)- Dr. Krishanpal Karmodiya. Associate Professor Department of Biology, IISER Pune
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(RM)- Rashim Malhotra. iPhD student Department of Biology, IISER Pune

1.Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites that are transmitted to humans by infected female Anopheles mosquitoes (https://www.cdc.gov/parasites/malaria/). In 2021, there were estimated 247 million cases of malaria worldwide and 619,000 deaths (WHO health report 2021) and the trend is increasing which is a grave concern (Fig. 1; Fig. 2) (https://www.cdc.gov/parasites/malaria/). It affects the productivity of life greatly as quantified from DALY's which shows in the age group of 15-45 a loss of 1.18 years (Gunda et al., 2016). Many temperate regions are also at risk of malaria as factors like global warming can create new niches for these parasites (Waite et al., 2019). Treatment for malaria uses antimalarial drugs such as artemisinin, but recently a rise in parasite drug resistance against these has been observed, which may lead to an increase in disease burden (Cowman et al., 2016). Therefore, to eradicate malaria in accordance with the sustainable development goals of the Government of India and the United Nations (https://www.un.org/sustainabledevelopment/health/), an alternative can be a surveillance system to track the infection, resulting in efficient containment of its spread and an early diagnosis scheme for treatment before its manifestation to the infectious stage. As a first step towards circumventing this, we therefore need to screen masses for clamping down its chain. Hence, it becomes imperative to diagnose infected individuals early on to reduce its damaging impact.

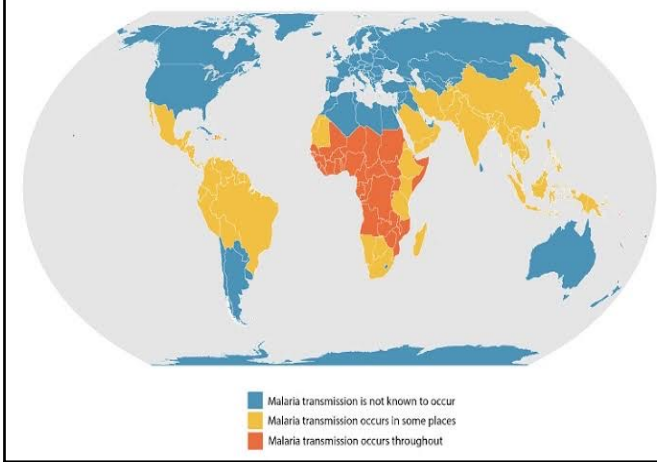


Fig. 1 Global Malaria transmission map 2022 (https://www.cdc.gov/malaria/about/)

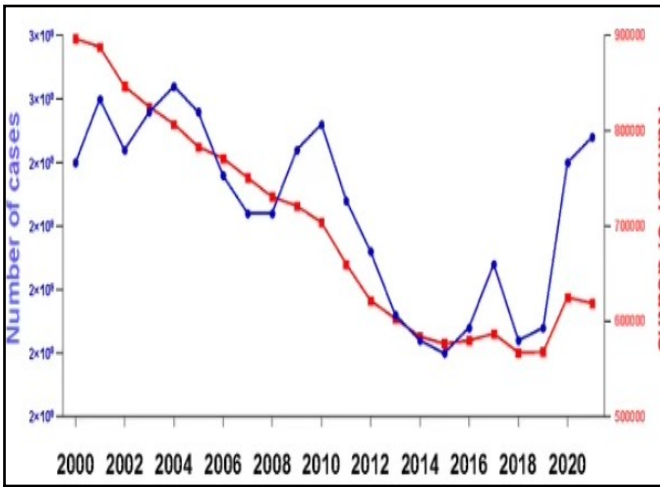


Fig. 2 Malaria case and deaths trend 2000-2020 (World Malaria health report 2022)

There are 5 species of *plasmodium* parasites (*P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*) which cause malaria to humans (Sato et al., 2021) . It follows a complex life cycle which involves 2 hosts mosquito and a human (Fig. 3). When an infected female anopheles mosquito takes a blood meal it transmits sporozoites into human blood. They travel through blood stream eventually infecting hepatocytes in liver. Upon maturation merozoites are released in blood stream which infect fresh RBC's and follow intra-erythrocytic stages of development. Starting from merozoite invasion of RBC's proceeding

towards intra-erythrocytic development in succession from ring, trophozoite and schizont stages (https://www.cdc.gov/parasites/malaria/ ; Crutcher et al., 1996). Further, mature schizonts ruptures RBC's, releasing 16-32 merozoites (Cowman et al ., 2016). Some merozoites after infecting RBC's also form gametes which can be taken up by mosquito during a blood meal. These intra-erythrocytic development cycles every 48 hours (https://www.cdc.gov/parasites/malaria/ ; Crutcher et al., 1996). It is in these stages of parasitic development clinical symptoms of malaria infection are visible.

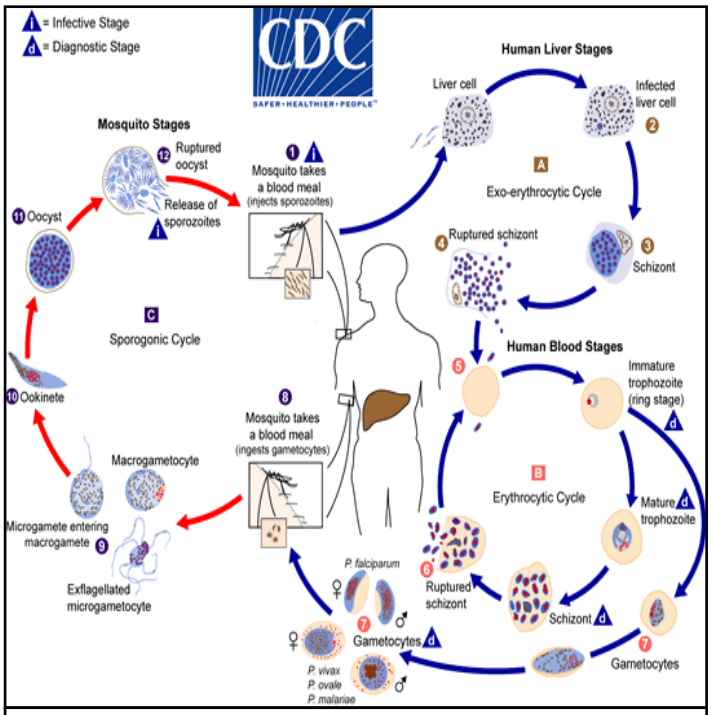


Fig. 3 Malaria Life cycle (https://www.cdc.gov/malaria/about/)

Nearly all diagnostic techniques target the intra-erythrocytic stage of infections. As it is in this stage they are most accessible for detection. Many detection techniques have been developed to check for infections but each method has its caveats (Table. 1). Microscopy by observing Giemsa-stained parasites in a blood sample is the current gold standard for malaria diagnoses, but is labour-intensive. Molecular diagnoses like qPCR/RT-qPCR targeting ribosomal and mitochondrial sequences, are quite accurate and sensitive but need investments in terms of equipment, trained manpower and processing time. On the other hand in an endemic region, serological detection is not reliable, since the rate of false positives is higher, as it also shows past exposure. On-field application of these techniques is limited, due to accessibility limitations of isolated or rural locations, where malaria is most prevalent. These techniques require infrastructure for disease detection, trained specialists, expenses, bulky equipment and also their maintenance capabilities. Antigen testing using rapid

Diagnosis method	Principle	Pros	Cons	LoD (Blood stage)
Clinical	Signs and symptoms	Helpful in detecting comorbidities	Late diagnosis, not accurate, for confirmation requires other validation techniques	Uncertain*
Field Microscopy	Staining of parasites	Gold standard for confirming parasite presence	Needs trained personnel	50 parasites/ µl*
Antigen detection	Antigens of parasite	Rapid detection and ease of use	Effective at high parasitemia, high false negativity	100 parasites/ µl*
Serological	Antibodies against parasites	Helpful to get population wise data of infection	False +ve results due to previous exposure, time required to generate antibodies	Uncertain*
Molecular (RTPCR/ PCR)	Parasite's nucleic acids	Highly sensitive, accurate	Needs elaborate set up, heavy investments of both time and expenditures	1-5 parasites/ µl°

*Table. 1 Comparative table for routinely used malaria diagnosis techniques *(Leski et al., 2020) °(Britton et al., 2016)*

diagnostic tests (RDTs) circumvents these logistical issues but is insensitive to early detection of infection. The reason is that they can detect parasitic infections after they reach a certain concentration. It targets highly expressing proteins viz. histidine-rich protein 2 (HRP-2), lactate dehydrogenase (pLDH) or *Plasmodium* aldolase (Moody ., 2002). Recent reports show that RDT testing is failing due to deletion in these regions of parasite antigens. Thus, further complicating diagnosis and resulting in false diagnosis of infection (Plucinski et al., 2021; Fola et al., 2023).

Thus these diagnostics schemes are challenging to implement for detection in symptomatic conditions the problem is complicated further when the patient is asymptomatic under low parasitemia or has other comorbidities.

Here we are attempting to develop a diagnostic method which can circumvent these drawbacks of the traditional detection techniques. Our objective is a detection technique which will conform to ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable to end-users) and hence has unrealized potential application (Ali et al., 2020). We focus on building a diagnostic scheme which is readily usable at point-of-care centres (POCs) with minimal training, expertise and resource requirements. That can be used for simultaneous detection in multiple samples.

We choose a diagnostic scheme based on nucleic acids, because of the flexibility with downstream assays that can be performed on them, lesser dependence on sophisticated instrumentation, and perishable reagents like antibodies required for readout. Isothermal amplification of nucleic acids (IANs) are simple reaction that has been developed for rapid, low-resource amplification. These IANs eliminate the need for thermocyclers since reactions are carried out at single temperatures requiring just a heating block. The sensitivity, time efficiency and amplification are better compared to PCR and RT-PCR (Zhao et al., 2015).

Of IAN's available viz. NASBA, ESDA, RPA, RCA and LAMP. Here we employ the LAMP reaction to develop a diagnostic scheme for malaria detection. Where LAMP stands for Loop Mediated Isothermal Amplification. This is a powerful nucleic acid amplification technique. Compared to other IANs this technique stands out overall in terms of extreme sensitivity, target specificity, excessive amplification products, shorter time requirements and relatively simple equipment/ reagents (viz. enzymes) required to carry out detection (Zhao et al., 2015). Schematic (Fig. 4) shows the working of LAMP reaction. Briefly, a set of 4 primers is needed (FIP, BIP, F3 and B3). On a dsDNA target sequence, invasion by forward inner primer

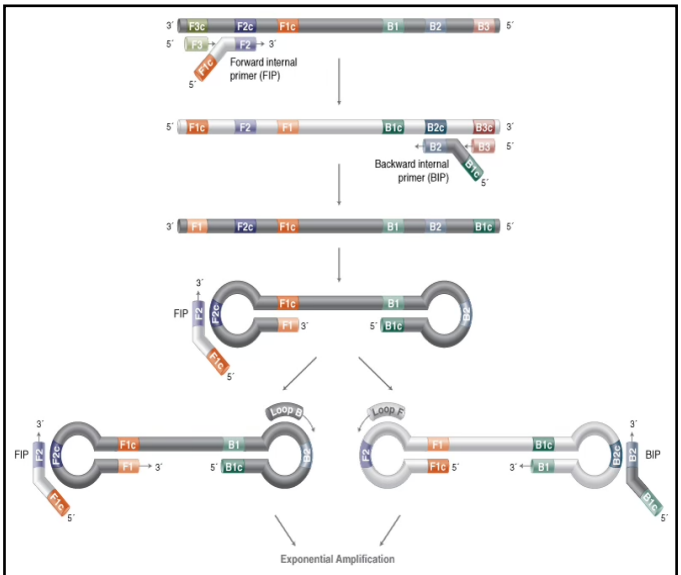


Fig. 4 LAMP reaction mechanism schematic (https://www.neb.com/en/applications/dna-amplification-pcr-and-qpcr/isothermal-amplification/loop-mediated-isothermal-amplification-lamp)

(FIP) occurs. These are then elongated by a strand displacing DNA polymerase. Next, the forward outer primer F3 displaces the elongated product of the inner primer, again with the help of a strand displacing polymerase. These displaced artifacts are then the template for the backward inner primer BIP, which is again displaced by its backward outer primer B3. This newly displaced amplicon is capable of forming dumbbell-like structures, due to the self-complementing design of the inner primer on itself after elongation. These dumbbell-shaped structures undergo exponential amplification in the presence of inner primers and produce concatemers of varying lengths with repeat stretches. The reaction takes place at a constant temperature (60-70 °C) and is completed within 30 min (Notomi et al., 2000; Ali et al., 2020). The necessity of, a strand displacing polymerase being, as there are no denaturation steps in the reaction which can displace amplicons for the next round.

Since RNA molecules have a higher abundance than DNA molecules, we are devising our kit to detect RNA molecules as well, thus increasing the sensitivity for detection. This requires an additional reverse transcription (RT) step. Modifying LAMP reaction to RT-LAMP. Thus a thermostable reverse transcriptase is needed which can operate under the reaction conditions of LAMP. This modification increases the sensitivity of the reaction and attains a lower detection limit compared to LAMP, which will leads to early-stage disease detection. To summarize, RT-LAMP reactions need 2 enzymes (reverse transcriptase, strand displacing DNA polymerase), RNA/DNA template and LAMP primers. The enzyme Reverse Transcriptase which converts RNA to cDNA and a strand displacing DNA polymerase amplifies cDNA. These enzymes are chosen such that they function optimally under similar conditions. To reduce the steps needed for completing the reaction.

LAMP amplification is huge, producing 11 µg of amplified product compared to 0.2 µg in the conventional PCR reaction (Shirato et al., 2019). Thus many detection techniques can be applied for validation viz;- Agarose gel electrophoresis, turbidity assays due to accumulation of magnesium pyrophosphate, colorimetric tests which are based on pH change or increasing Mg ions in the reaction, fluorescent dyes which fluoresce in the presence of LAMP amplicons (dsDNA). These are indirect methods of detection which can be employed simplistically.

For higher specificity, we are focusing our detection on a sequence-directed approach. CRISPR-based modules (Fig. 5) have been utilized for this purpose (Tian et al., 2020; Nguyen et al., 2022). A stretch of the sequence is targeted using a thermostable Cas enzyme (eg: Cas12b family) and sgRNA. These enzymes are chosen to work under RT-LAMP reaction conditions. They have indiscriminate trans-cleavage activity targeting ssDNA and are activated only upon recognition and binding of its sgRNA to its complimentary dsDNA, adjacent to the PAM site (Tian et al., 2020). This sequence-based activity is

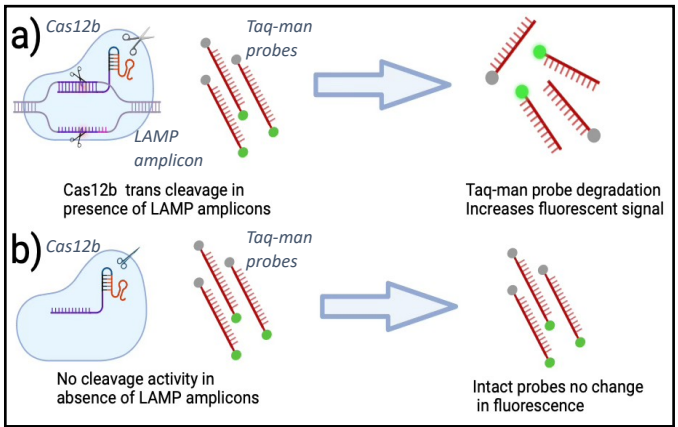


Fig. 5 Cas12b fluorescence based detection scheme

exploited to target LAMP amplicons. In the presence of modified ssDNA oligomers, a readout for the detection scheme is generated. These modified probes are tagged with a Fluorophore-Quencher at the 2 ends (Molecular Beacons). The readout in this kind of RT-LAMP-CRISPR system is an increase in fluorescence signals which takes place only in the presence of target RNA on its conversion to dsDNA molecules. The Cas enzyme on activation by LAMP amplicons cleaves off the molecular beacon increasing the fluorescent readout in the reaction. This kind of reporter assay for parasite detection needs a light source and a detector. To further simplify the readout, a Lateral Flow Assay using a dipstick is also readily usable (Fig. 6). Where different striping patterns between negative and positive samples give a straightforward visual status. Similar to molecular beacons a modified ssDNA probe with FAM and Biotin at ends is used. The dipstick is coated with 2 different molecules that traps FAM and Biotin on separate regions of the strip. FAM is immobilized indirectly in the presence of anti-FAM antibody gold nanoparticles (present in LFA running buffer), on the strip by antibodies targeting anti-FAM antibodies. Biotin is immobilized by streptavidin fixed on the strip. When the probes are cleaved clear striping is visible at the test line in a positive reaction. Else a band is visible at the control line where probes are blocked by streptavidin. The bands are visible due to the accumulation of gold nanoparticles at specific a locations creating surface plasmon resonance (Fig. 6).

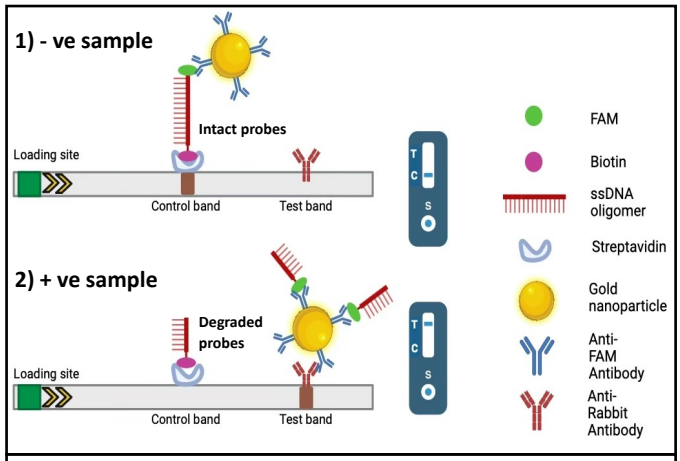


Fig. 6 Lateral flow strip based detection scheme

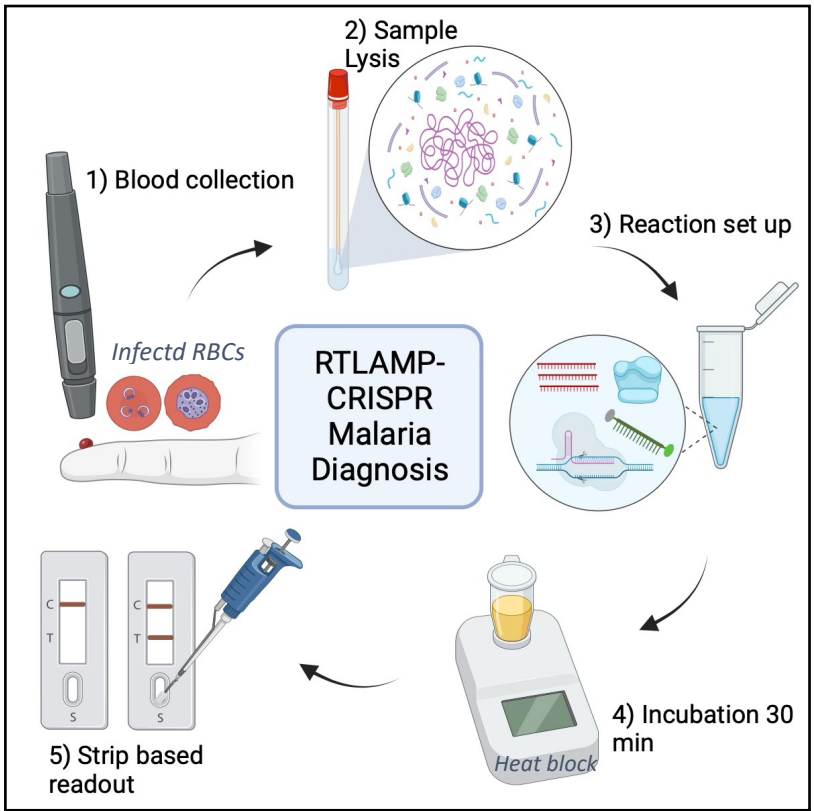


Fig. 7 Schematic for RT-LAMP-CRISPR based detection module

Overall RT-LAMP coupled CRISPR detection module will be a one-pot diagnosis kit (Fig. 7) which will eliminate the drawbacks of conventional diagnosis methods. It will be field deployable user friendly, relatively cheaper, fast, sensitive and accurate.

2. Materials and Methods

2.1. Analyzing RNA sequencing data

RNA sequencing analysis is performed on Galaxy servers. RNA sequencing data is obtained from (Tonkin et al., 2018; Bruhad et al., 2022). The database is available on the European Nucleotide Archive (ENA) and Gene Expression Omnibus (GEO). The sample study accession numbers are PRJEB21707 and GSE179055. The following are the run accession numbers used for data analysis:- ERR2028864, ERR2028863, ERR2028862, ERR2028861, ERR2028860, ERR2028859, ERR2028858, SRR14931119, SRR14931120, SRR14931121, SRR14931122, SRR14931123, and SRR14931124.

The workflow applied on the RNA seq. data set is given below:
-Data is uploaded to Galaxy servers using 'Faster download and Extract reads in FASTQ format from NCBI SRA'
-Quality check of raw reads using 'FastQC'
-Raw sequencing reads were preprocessed for adapter trimming and removing low-quality reads using 'Trimmomatic'
-Cleaned reads were mapped to PlasmoDB.v65 using 'HISAT2'
-Quantification of gene expression levels is done by their FPKM values using 'Cufflinks'
-The FPKM values generated here are used for transcript selection based on abundance by sorting.
-PCA plot is generated using 'DeSeq2' and 'Feature Counts'.

2.2. RT-LAMP primer designing

We used NEB's LAMP primer design tool (https://lamp.neb.com/#!/) to design primers. For selected transcripts, a spliced mRNA sequence from PlasmoDB is obtained and subjected to further constraints explained in sections (3.2) and (3.3). These screened transcripts or regions of the transcript are used for primer designing. The following parameters (Table. 2) are set for LAMP primer design.

Primer Design Parameters		
General	Sorting Rule	Easy
Reaction Conditions	Na^{+} concentration	50.0 mM
	Mg^{++} concentration	8.0 mM
Lengths (min/max)	F1c/B1c	20 nt - 22 nt
	F2/B2	18 nt - 20 nt
	F3/B3	18 nt - 20 nt
	LF/LB	15 nt - 25 nt
T_m (min/max)	F1c/B1c	64 °C - 66 °C
	F2/B2	59 °C - 61 °C
	F3/B3	59 °C - 61 °C
	LF/LB	64 °C - 66 °C
% GC	Min/Max	40 % - 65 %
	Min/Max (Loop)	40 % - 65 %
ΔG threshold	5′ Stability	-4 Kcal/mol
	3′ Stability	-4 Kcal/mol
	3′ Stability (Loop)	-2.0 Kcal/mol
	Dimer check	-2.5 Kcal/mol
	Dimer check (Loop)	-3.5 Kcal/mol
Distances (min/max)	(F2-B2)	120 nt - 160 nt
	Loop (F1c-F2)	40 nt - 60 nt
	F2-F3	0 nt - 60 nt
	F1c-B1c	0 nt - 100 nt
Limits	F1c/B1c	3
	F2/B2	10
	F3/B3	3
	LF/LB	10
	Sets	1000

Table. 2 RT-LAMP primer design parameters

2.3. Enzyme purification

We received plasmids (pET_RTX-6xHis-#145029 and pET21a(+)-BstLF-6xHis-#159148) from Addgene. The plasmids are transformed in Bl21 DE3 competent cells for protein expression. We transformed 1 µg plasmids into these cells. By thawing for 30 min together on the ice. Next, a heat shock of 42 °C for 90 sec and a cold shock of 0 °C for 10 min is applied. 1ml Luria Broth (LB) is added to the tubes and incubated for 1 hour at 37 °C, at 180 rpm. Then the cells were centrifuged at 3500 rpm for 5 min and the supernatant was discarded. The cells were plated on a Luria agar (LA) plate with the antibiotic-resistant marker (1:1000 ratio by volume of Antibiotic to LA, Ampicillin concentration 50 mg/ml) and incubated at 37 °C for 12 hours. Next, a single colony is picked up and expanded in primary LB culture 10 ml with an antibiotic marker (1:1000 ratio of Antibiotic to culture volume) at 37 °C, 180 rpm for 12 hours. This primary culture is expanded to the secondary culture of 1 liter LB (antibiotic resistant marker in 1:1000 ratio by volume) till the OD is in the range of 0.6-0.8 at 37 °C, 180 rpm. To express the proteins in the cells IPTG (1 M) induction is carried out. For 1 liter culture, 500 µl of IPTG is added and the culture is further incubated at 18 °C, 16-18 hrs at 180 rpm. The culture is next centrifuged at 4000 rpm, 15 min at 4 °C. Supernatant is discarded and the bacterial pellet resuspended in 1xPBST (NaCl 137mM, KCl 2.7mM, Na2HPO4 10mM, KH2PO4 1.8mM, Tween20 0.1%), and transferred to fresh falcon. Culture is again centrifuged for 15 min, 4000 rpm, and 4 °C, the supernatant is discarded, and the pellet is stored at -80 °C.

The pellet is thawed on ice. Next, 30 ml of Lysis Buffer is added to dissolve it. Using a sonicator, cells are lysed at 40 % amplitude, at a pulse rate of 1 sec ON and 4 sec OFF, with a 2 min total ON cycle. The lysate is centrifuged at 12000 rpm at 4 °C for 60 min. The lysate is transferred into a new falcon. It is then heat treated at 70 °C for 10 min and placed on ice for 10 min. Centrifuge the heat-treated lysate at 12000 rpm, 4 °C for 50-60 min. Filter the supernatant lysate through a 0.2 µm filter. Since we have the enzymes which are histidine-tagged. We do IMAC using Ni-Agarose beads. For this 1 ml of bead slurry is used for 1-liter culture in a fresh falcon (50 ml). It is centrifuged for 2 min, at 2000 rpm, 4 °C. The supernatant is discarded, and the beads are washed with 10 ml autoclaved-miliQ water by re-suspending and centrifuging for 2 min, 2000 rpm at 4 °C then discard the supernatant. Equilibrate beads with 10 ml equilibration buffer by re-suspending the beads and again discarding the supernatant after centrifuging at 2000 rpm, 2 min at 4 °C, and repeat the above steps twice. Transfer the lysate to the bead falcon and set it up for binding on the roller at 4 °C for 5 hours. Centrifuge the beads for 30 min, 12000 rpm, 4 °C, and store the supernatant separately. Give washes to the beads with wash buffer (1.5ml) by re-suspending. Incubate the beads on ice for 5 min. Next centrifuge the beads for 3 min, 2000 rpm at 4 °C. Separate and store the supernatant without disturbing the bead layer. Repeat the washes 5 times. Next using elution buffer (1.5 ml) resuspend the beads incubate on ice for 5 min, centrifuge for 3 min, 2000 rpm at 4 °C, and collect the supernatant. Repeat the elution steps to collect 5 elution. Pool the elutions together and set up for dialysis in snakeskin dipped in dialysis buffer (1 liter) at 4 °C with a magnetic stirrer at 180 rpm. Change the dialysis buffer after 5 hours and keep it for dialysis for 7 hours again. Collect the dialyzed elution and store the purified enzyme at -20 °C.

For purification of Reverse Transcription Xenopolymerase (RTx) and Geobacilllus Steareothermophilus polymerase 1 large fragment (Bst lf). We referred the purification protocols from (Bhadra et al., 2020) for RTx and (Milligan et al., 2018) for Bst lf.

Protein purification buffer composition tabels:

Buffer	Components	Buffer	Components	Buffer	Components
Lysis Buffer (30mL)		**Equilibration Buffer (20ml)**		**Elution Buffer (10mL)**	
	Phosphate Buffer-50mM - 7.5pH		Phosphate Buffer-50mM - 7.5pH		Phosphate Buffer-50mM - 7.5pH
	NaCl - 300mM		NaCl - 300mM		NaCl - 300mM
	Imidazole - 10mM		Imidazole - 10mM		Imidazole - 150mM
	MgSO4 - 5mM				
	Igepal CO-630 (non-toxic Non-idet P40 equivalent) - 0.1%	**Wash Buffer (10ml)**		**Ni-NTA Dialysis Buffer (2L)**	
	EDTA-free protease inhibitor tablet - 1x		Phosphate Buffer-50mM - 7.5pH		Tris-HCl- 40mM - 7.5pH
	HEW Lysozyme - 30-60 mg in 30mL		NaCl - 300mM		NaCl- 100mM
			Imidazole - 20mM		DTT- 1mM
					Igepal CO-630 (non-toxic Non-idet P40 equivalent) - 0.1%

Table. 3 RTx purification buffer composition

Buffer	Cmponents	Buffer	Components	Buffer	Components
Lysis Buffer (30mL)		**Wash Buffer (10ml)**		**Ni-NTA Dialysis Buffer (2L)**	
	TrisHCl- 20 mM-7.4pH		Tris-HCl- 20mM		Tris-HCl- 10mM
	NaCl- 300mM		NaCl- 300mM		KCl- 100mM
	Imidazole- 10mM		Imidazole- 20mM		DTT- 1mM
	Tween20- 0.1%		Tween-20- 0.1%		EDTA- 0.1mM
	EDTA-free protease inhibitor tablet		EDTA-free protease inhibitor tablet		Glycerol- 50%
	Lysozyme- 0.5mg/ml		Lysozyme- 0.5mg/ml		Tween20- 0.5%
					TritonX100- 0.5%
Equilibration Buffer (20ml)		**Elution Buffer (10mL)**			
	Tris-HCl-20mM-7.4pH		Tris-HCl- 20mM		
	NaCl- 300mM		NaCl- 300mM		
	Imidazole- 10mM		Imidazole- 150mM		
	Tween-20- 0.1%		Tween-20- 0.1%		
	EDTA-free protease inhibitor tablet		EDTA-free protease inhibitor tablet		
	Lysozyme- 0.5mg/ml		Lysozyme- 0.5mg/ml		

Table. 4 Bst lf purification buffer composition

2.4. Parasite pellet harvest

Parasite pellet is isolated from infected RBC (iRBC) culture. The culture is transferred from the cell culture flask to the falcons. Centrifuge the falcon for 10 min at, 4000 rpm, 4 °C. Discard the supernatant and add 10 ml 1x phosphate buffer saline (PBS) (NaCl 137mM, KCl 2.7mM, Na2HPO4 10mM, KH2PO4 1.8mM) to 30 ml culture. Re-suspend iRBC in PBS and centrifuge for 5 min, 4000 rpm at 4 °C. Discard the supernatant. Make saponin solution of 50 ml solution (0.08 gram saponin in 50 ml 1xPBS). For 30 ml culture which is pelleted above add 7 ml saponin solution. Vortex the pellet for 15 seconds. Incubate at 37 °C for 15 min. Vortex the mix for 15 seconds and add 3ml 1xPBS to it. Centrifuge the mix at 6000 rpm for 15 min at 4 °C. Discard the supernatant. Add 1ml 1xPBS to the pellet. Transfer it to a 1.5 ml micro centrifuge tube. Centrifuge at 14000 rpm for 2 min at 4 °C. Discard the supernatant. Give 1 ml 1xPBS washes by re-suspending the pellet and centrifuging at 14000 rpm, 4 °C for 2 min. Discard the supernatant. Repeat the wash step till the supernatant is clear. Generally, it takes 3 to 4, 1xPBS washes. Store the pellet in -80 °C. Try to remove the Buffy coat on the pellet which appears on top of the parasite pellet during washes while discarding the supernatant.

2.5 RNA isolation from Parasite pellet

Dissolve the pellet in trizol reagent (5x the volume of the pellet). Re-suspend till the pellet is completely broken and uniformly mixed. Incubate it at 22 °C for 5 min. Spin down the pellet at 12000 rpm for 7 min at 4 °C. Add 0.2 volume of chloroform relative to the trizol added. Invert and mix the tube 15 times gently. Incubate it at 22 °C for 12 min. In pre-chilled centrifuge at 4 °C, centrifuge for 12 min at 12000 rpm. This creates 3 layers in the tube. The top layer is a clear aqueous phase, the middle layer is interphase, and the bottom layer organic phase. Gently elute the aqueous phase without disturbing the interphase. Transfer it to a new tube. Add the same amount of chloroform to this again and centrifuge the tube at 12000 rpm, 12 min at 4 °C. Transfer the aqueous phase again in a new micro centrifuge tube. Add 0.8 volume of isopropanol relative to the aqueous layer. Add 3 µl glycogen and mix the solution thoroughly. Incubate the solution for 12 hours at -20 °C. Centrifuge the tube for 50 min, 4 °C at 12000 rpm. Discard the supernatant and give 75% ethanol wash of 500 µl, thrice by spinning at 12000 rpm, 4 °C for 15min. Discard the supernatant after each wash. Incubate the pellet at room temperature till the Ethanol evaporates. Do not let the RNA pellet over-dry. Add nuclease-free water to the pellet 15-20 µl. Store the pellet in -80 °C.

2.6 RNA isolation directly from infected RBCs

The protocol for isolation of RNA directly from iRBCs is similar to RNA isolation from parasite pellet. Spin down the parasite culture and discard the supernatant. Proceed with the steps from section (2.5).

2.7 cDNA synthesis using Promega kit

The cDNA synthesis is performed using the Promega Reverse Transcription kit. The concentration of RNA is quantified using Thermofischer Nanodrop. Protocol for cDNA synthesis:

1- DNAse treatment-
Incubate the reaction (Table. 5) at 37 °C for 1 hour.

Add 1 µl DNAse stop solution and incubate the reaction vial at 65 °C for 20 min.

Reagent	Amount (10 µl)
10x DNAse buffer	1 µl
DNAse Enzyme	1 µl
RNA	1 µg
NFW	Enough to make 10 µl reaction

Table. 5 DNAse treatment reaction reagents

2- Primer annealing-
Add 1 µl Random Hexamers to above reaction, and incubate at 70 °C for 10 min. Transfer the reaction on ice slurry immediately.

Reagent	Positive reaction	Negative control
12 µl	**Amount**	**Amount**
RT buffer	4 µl	4 µl
MgCl2	3 µl	3 µl
dNTP (10mM)	1 µl	1 µl
Reverse Transcriptase	1 µl	0 µl
RNA template	6 µl	6 µl
NFW	5 µl	6 µl

Table. 6 Reverse transcription reaction reagents for positive reaction and negative control

3- Setting up reverse transcription reaction-
Split the total 12 µl reaction in 2 tube equally (6 µl). Set up reaction as follows (Table. 6).

In a thermal cycler run the reactions at 25 °C- 5 min, 37 °C- 60 min, 70 °C- 10 min and hold at 4 °C. Verify cDNA synthesis by doing a PCR using appropriate primers for both reaction. Analyze the PCR products by Agarose Gel Electrophoresis.

2.8 Reverse Transcription using RTx polymerase

Following reagents are used in setting up reverse transcription reaction. The reaction is incubated at 68 °C for 30 min for cDNA synthesis.

Reagent	Amount for 25 µl reaction
10x Isothermal buffer	2.5 µl
(NH4)2SO4 - 100 mM	2 µl
dNTP 10 mM	1 µl
RTX	40 ng
Random Hexamers	1 µl
RNA	0.5 µg
NFW	Make volume 25 µl

Table. 7 Reverse transcription reaction reagents using RTx polymerase

10x Isothermal Buffer components	Amount
Tris-HCl (pH8.3)	250 mM
MgCl2	15 mM
KCl	375 mM
DTT	100 mM

Table. 8 Isothermal buffer composition for RTx polymerase reverse transcription reaction

2.9 LAMP reactions using Bst lf

The LAMP reaction performed using Bst lf is set up at 65 °C for 40 min. Following are the reaction components for LAMP reactions.

The RT-LAMP assays using RTx / NEB RTx enzymes is modified with addition (40 ng / 0.2 µl) of enzyme in reaction and RNA as template instead of DNA.

Components	Amount for 25 µl reaction
10x Isothermal Buffer	2.5 µl
MgSO4 100 mM	1.13 µl
dNTP 10 mM	1.4 µl
FIP/BIP 10 mM	4 µl
F3/B3 10 mM	1 µl
Bst Lf	1 µg
cDNA/ PCR amplicon	0.5 µg
NFW	Make volume 25 µl

Table. 9 LAMP reaction reagents using Bst lf

10x Isothermal Buffer components	Amount
Tris-HCl (pH8.8)	200 mM
(NH4)2SO4	100 mM
MgSO4	20 mM
KCl	500 mM
Tween 20	1%

Table. 10 Isothermal buffer composition for Bst lf LAMP reaction

2.10 RT-LAMP reactions using NEB RT-LAMP kit

The RT-LAMP reaction using NEB RT-LAMP kit (catalog no. #M1708S) is performed on RNA template by incubating reaction reagents at 64 °C for 40 min.

Following is the reagent (Table. 11)

RT-LAMP reaction component	Amount for 10 µl reaction
RT-LAMP kit	5 µl
FIP / BIP (10 mM)	1.6 µl
F3 / B3 (10 mM)	0.4 µl
RNA	1 µl
NFW	Make up 10 µl

Table. 11 RT-LAMP reaction reagents using NEB kit

2.11 Parasite culture

The parasites were cultured in RPMI1640 medium supplemented with 25 mM HEPES, 0.5% AlbuMAX I, 1.77 mM sodium bicarbonate, 100 µM hypoxanthine, and 12.5 µg / ml gentamicin sulfate at 37 °C (Radfar et al., 2009). Hematocrit was maintained at 3% using O-positive human RBC. Parasitemia was monitored using Giemsa-staining of a thin blood smear and calculated by observing 10 fields.

Dilution for infected RBCs (iRBCs) was created by mixing fresh O positive human RBC (PCV) with iRBCs of known parasitemia. 200 µl of total RBC is used for RNA extraction by mixing iRBC and RBC in ratios, for desired dilutions

3.Results

3.1) RNA sequencing data set procurement

We started by analyzing transcript datasets from different sources. We obtained RNA seq data of field samples from (Tonkin et al., 2018). We also analyzed RNA seq data from (Bruhad et al., 2022), here RNA seq is performed on parasite culture under different conditions like temperature stress, and artemisinin drug stress. On analyzing these data sets we found that field samples are highly variable (Fig. 8). The culture-grown parasites do not mimic the field condition. Implying detection will be robust if we select transcripts from field samples as it will be a reflection of real-world conditions.

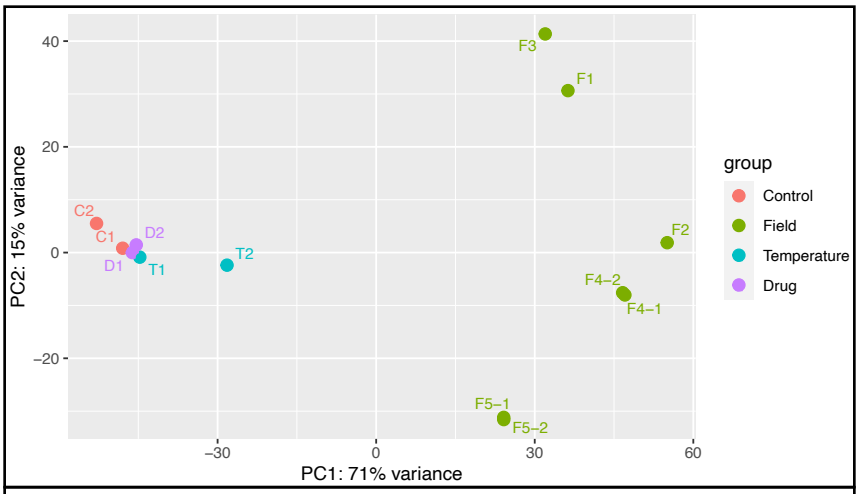


Fig. 8 PCA plot for RNA seq data from different samples

3.2) Target screening

To select transcripts, the data was analyzed according to the workflow provided in the methods (2.1). Transcript abundance of P. falciparum in field conditions is quantified using FPKM values. Following constraints were applied to select target genes, on which primers are designed for RT-LAMP assays.

a) The transcripts were sorted according to their FPKM values from highest to lowest, and only transcripts with the highest expression were chosen.
b) Size selection of transcripts was performed, excluding transcripts that were smaller than 100 bp.
c) Since the diagnostic module needs to be specific to P. falciparum we checked for sequence uniqueness by performing 'NCBI blastn' alignment on a non-redundant nucleotide database using NCBI blast search. Only those transcripts were considered which shared no similarity with human transcripts and other organisms as well.
These are the pre-screened target transcripts (Table. 12).

PlasmoDB ID	Description	Blastn (against Humans)
PF3D7_0532100	Early Transcribed Membrane Protein 5	No similarity
PF3D7_0210100	60S Ribosomal Protein L37ae, putative	1:150-190:250 no similarity
PF3D7_1121600	Exported Protein 1	No significant similarity
PF3D7_1462800	Glyceraldehyde-3-phosphate Dehydrogenase	1:300 no similarity
PF3D7_1132800	Aquaglyceroporin	No significant similarity
PF3D7_0206800	Merozoite Surface Protein 2	No significant similarity
PF3D7_0202000	Knob-Associated Histidine-Rich Protein	400:1965 no similarity
PF3D7_1102800	Early Transcribed Membrane Protein 11.2	140:285 no similarity
PF3D7_0102200	Ring-Infected Erythrocyte Surface Antigen	Discontinuous overlap with 60 bp max
PF3D7_0935900	Ring-Exported Protein 1	1:522 - 830:2142

Table. 12 Prescreened target transcripts of P. falciparum from clinical samples

3.3) Primer design

From the preselected transcripts we looked at the splicing events across the transcripts in Integrated Genome Viewer (IGV). We choose the regions which were devoid of splicing events and designed LAMP primers on such regions using NEB's LAMP primer designer. On some of the prescreened transcripts we were unable to design primers. These constraints further screened targets. Based on this we were left with the following set of transcripts (Table. 13).

PlasmoDB ID	Description
PF3D7_0532100	Early transcribed membrane protein 5
PF3D7_1121600	Exported protein 1
PF3D7_1462800	Glyceraldehyde-3-phosphate dehydrogenase
PF3D7_1102800	Early transcribed membrane protein 11.2
PF3D7_0102200	Ring-infected erythrocyte surface antigen

Table. 13 Target transcripts of P. falciparum from clinical samples

The following are the regions in sequences of selected transcripts (Table. 13) where LAMP primer are designed.
F3 / B3 primer sequences are green in color
F1c / B1c primer regions blue in color
F2 / B2 sequence are highlighted in red
F1c-F2 sequence are attached together by phosphodiester bond making it FIP primer, similarly the B1c-B2 make up the BIP primer

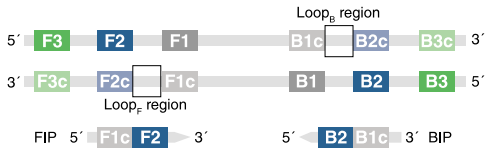
i) Gene :- Early transcribed membrane protein 5
Gene ID :- PF3D7_0532100
Sequence :-

ATGAGATTCTCCAAAGTATTTTCTTTTTTCGCCTTTTTCATTGCCCTTAAATATTTTAACAGATGCCTTGGTGATCAATTAGATATGGGTTCCGTACACAATAACAATTCCGTTGT
AGGAAACTCATCATCACATTCACCATCATCATCATCATCTTCACCATCTTCTTCTTCTTCTTCATCATCATCATCACCATCTGCATCTTCATCTTCATCCTCATCATCCCCAGCTT
CCTCATCTTCAAGCCCATCAAGCACATCAGATGACAGCAAAAATGCATCCTTAGATAAAATCGATGAAGAACTCCAAAAGAAAAAGAAAAACGAAAAATTACTTTTAATAT
CCTCTATTGCCACAGGTTTAGCCGTTTTAGTAGGTGGAATAATAGGTACTGCTTTATACGCAAGCAGAAAATCATCAAAAGCCTGCAAAAATAACGTTGACGATTTAGATTCA
GATGTTGAAGAAGCCGATGTTACCGAGGAATCAGCTGTTGAAACCAAGAAAGACGAAGTCAAAACCGAAGAACCCAAAAAAGAACAATAA

Target: PF3D7_0532100, subsequence 230-546

Selected Primer Sets

Required primers:
F3
B3
FIP - Forward Internal Primer
BIP - Backward Internal Primer
LF - Loop Forward Primer
LB - Loop Backward Primer



SetID: PF3D7_0532100_P14L1

	5′ pos	3′ pos	len	Tm	5′ dG	3′ dG	% GC	(required oligos are orange)
F3	75	97	23	56.00	-4.86	-4.85	30	CTCCAAAAGAAAAAGAAAAACGA
B3	250	267	18	56.87	-4.66	-6.47	56	CAACAGCTGATTCCTCGG
FIP			50					AGCAGTACCTATTATTCCACCTACTTACTTTTAATATCCTCTATTGCCAC
BIP			46					ATACGCAAGCAGAAAATCATCAAAATAACATCGGCTTCTTCAACAT
LF								
LB	195	217	23	64.44	-7.09	-5.60	43	GCCTGCAAAAATAACGTTGACGA
F2	103	127	25	56.41	-3.24	-6.41	32	TACTTTTAATATCCTCTATTGCCAC
F1c	143	167	25	60.61	-5.63	-4.41	40	AGCAGTACCTATTATTCCACCTACT
B2	229	249	21	57.26	-3.29	-4.21	38	TAACATCGGCTTCTTCAACAT
B1c	170	194	25	60.24	-5.30	-3.69	32	ATACGCAAGCAGAAAATCATCAAAA

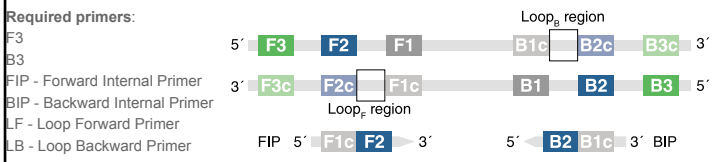
Fig. 9 LAMP primers for Early transcribed membrane protein 5

ii) Gene :- Exported protein 1
Gene ID :- PF3D7_1121600
Sequence :-

ATGAAAATCTTATCAGTATTTTTTCTTGCTCTTTTCTTTATCATTTTCAATAAAGAATCCTTAGCCGAAAAAACAAACAAAGGAACTGGAAGTGGTGTTAGCAGCAAAAAAA
AAAATAAAAAAGGATCAGGTGAACCATTAATAGATGTACACGATTTAATATCTGATATGATCAAAAAAGAAGAAGAACTTGTTGAAGTTAACAAAAGAAAATCCAAATATAA
ACTTGCCACTTCAGTACTTGCAGGTTTATTAGGTGTAGTATCCACCGTATTATTAGGAGGTGTTGGTTTAGTATTATACAATACTGAAAAAGGAAGACACCCATTCAAAATAG
GATCAAGCGACCCAGCTGATAATGCTAACCCAGATGCTGATTCTGAATCCAATGGAGAACCAAATGCAGGCCCACAAGTTACAGCTCAAGATGTTACACCAGAGCAACCAC
AAGGTGACGACAACAACCTCGTAAGTGGCACTGAACACTAA

Target: PF3D7_1121600, subsequence 1-489

Selected Primer Sets



SetID: PF3D7_1121600_P26L1

	5′ pos	3′ pos	len	Tm	5′ dG	3′ dG	% GC	(required oligos are orange)
F3	270	290	21	55.02	-4.06	-5.17	43	CGTATTATTAGGAGGTGTTGG
B3	471	489	19	55.91	-3.74	-5.00	42	TTAGTGTTCAGTGCCACTT
FIP			44					ATCTGGGTTAGCATTATCAGCTGGAAAAAGGAAGACACCCATTC
BIP			38					TGGAGAACCAAATGCAGGCCCGAGGTTGTTGTCGTCAC
LF								
LB	420	442	23	64.48	-5.49	-5.59	48	AGCTCAAGATGTTACACCAGAGC
F2	310	330	21	56.57	-3.29	-4.51	43	GAAAAAGGAAGACACCCATTC
F1c	350	372	23	60.12	-4.74	-5.31	43	ATCTGGGTTAGCATTATCAGCTG
B2	452	469	18	57.29	-6.02	-5.84	56	CGAGGTTGTTGTCGTCAC
B1c	390	409	20	62.74	-5.11	-6.69	55	TGGAGAACCAAATGCAGGCC

Fig. 10 LAMP primers for Exported Protein 1

iii) Gene :- Glyceraldehyde-3-phosphate dehydrogenase

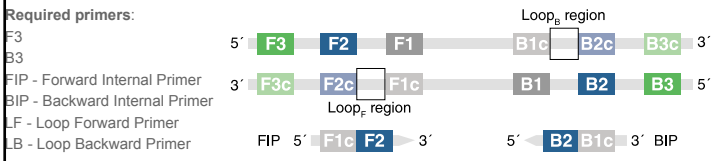
Gene ID :- PF3D7_1462800

Sequence :-

ATGGCAGTAACAAAACTTGGAATTAATGGATTTGGTCGTATCGGACGTTTAGTATTTAGAGCAGCCTTTGGAAGGAAAGATATCGAAGTAGTTGCTATTAACGACCCATTTAT
GGACCTTAACCACTTATGCTATCTCTTGAAATACGATTCAGTACATGGTCAATTTCCATGTGAGGTAACCCACGCTGATGGATTTTTATTAATCGGAGAAAAGAAAGTCAGTG
TTTTTGCTGAAAAGGATCCATCTCAAATTCCTTGGGGAAAATGCCAAGTAGATGTTGTATGTGAATCAACTGGTGTATTTTTAACCAAAGAATTAGCTAGCAGTCACCTTAAG
GGAGGAGCCAAGAAGGTTATTATGTCCGCCCCACCAAAGGATGACACCCCAATTTATGTTATGGGTATTAACCACCACCAATATGATACCAAACAACTTATTGTTTCCAATGC
ATCATGTACCACAAACTGCTTAGCTCCATTAGCTAAAGTTATTAATGATCGTTTTGGAATTGTTGAAGGTTTAATGACCACCGTTCATGCATCCACTGCTAACCAATTAGTTGT
TGATGGTCCATCAAAGGGTGGTAAGGACTGGAGAGCAGGTAGATGTGCATTATCCAACATTATTCCAGCTTCCACTGGTGCAGCTAAAGCTGTAGGTAAAGTTTTACCTGAA
CTTAATGGAAAATTAACAGGTGTAGCTTTCAGAGTACCAATTGGAACTGTATCAGTTGTTGATTTAGTTTGCAGATTACAAAAACCAGCAAAATATGAAGAAGTTGCTTTAG
AAATTAAAAAAGCTGCTGAAGGTCCACTTAAAGGAATCTTAGGATACACTGAAGATGAAGTTGTTTCTCAAGATTTCGTTCATGATAACAGATCATCAATCTTTGACATGAA
AGCTGGTTTAGCCTTAAACGACAATTTCTTCAAATTAGTTTCATGGTACGATAATGAATGGGGATACTCAAACCGTGTTCTTGATTTAGCCGTACACATTACTAACAACTAA

Target: PF3D7_1462800, subsequence 700-1014

Selected Primer Sets



SetID: PF3D7_1462800_P1

	5′ pos	3′ pos	len	Tm	5′ dG	3′ dG	% GC	(required oligos are orange)
F3	10	30	21	55.71	-3.82	-4.13	38	AGAGTACCAATTGGAACTGTA
B3	205	223	19	55.75	-4.18	-6.24	47	CGTTTAAGGCTAAACCAGC
FIP			49					GGACCTTCAGCAGCTTTTTTAATTTCAGTTGTTGATTTAGTTTGCAGAT
BIP			48					AGGATACACTGAAGATGAAGTTGTTTCATGTCAAAGATTGATGATCTG
LF								
LB								
F2	32	55	24	57.99	-4.66	-5.14	33	CAGTTGTTGATTTAGTTTGCAGAT
F1c	92	116	25	60.70	-5.69	-2.40	36	GGACCTTCAGCAGCTTTTTTAATTT
B2	180	202	23	56.37	-4.46	-4.25	35	TCATGTCAAAGATTGATGATCTG
B1c	132	156	25	60.06	-3.82	-4.32	36	AGGATACACTGAAGATGAAGTTGTT

Fig. 11 LAMP primers for Glyceraldehyde-3-phosphate dehydrogenase

iv) Gene :- Early transcribed membrane protein 11.2

Gene ID :- PF3D7_1102800

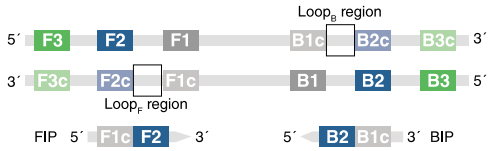
Sequence :-

ATGAAAATCACAAAGATCTTTTATTTCTTCGCCGCCTTATTGGCCTTAAACTTCATTGCCCCAAACTACTTTAACGGATATGTAGAAGCAAAGAAAGCTTTAACACCAGCTGA
AAAGAAAAAGAGAAACCAACAAATCATGTTAATCTCTGGTATTACCTCAGCTTTAGCACTTCTCATTGGTGCTGGTGTAGGTCTCGGAATCCACTACAAGAACAAGAATAAC
GGTGATGAAAAGAAAGATAAAGCAGGAGCAAAAACCATCACCGCCACTCCAAAAAACTAA

Target: PF3D7_1102800, subsequence 1-283

Selected Primer Sets

Required primers:
F3
B3
FIP - Forward Internal Primer
BIP - Backward Internal Primer
LF - Loop Forward Primer
LB - Loop Backward Primer



SetID: PF3D7_1102800_P19

	5′ pos	3′ pos	len	Tm	5′ dG	3′ dG	% GC	(required oligos are orange)
F3	35	55	21	55.44	-3.57	-4.41	38	CCTTATTGGCCTTAAACTTCA
B3	222	243	22	55.40	-3.40	-5.02	32	ATCTTTCTTTTCATCACCGTTA
FIP			45					TCTCTTTTTCTTTTCAGCTGGTGTTTGCCCCAAACTACTTTAACG
BIP			45					TCTCTGGTATTACCTCAGCTTTAGCGTAGTGGATTCCGAGACCTA
LF								
LB								
F2	57	76	20	57.55	-7.20	-4.18	45	TGCCCCAAACTACTTTAACG
F1c	102	126	25	61.37	-4.10	-5.16	36	TCTCTTTTTCTTTTCAGCTGGTGTT
B2	191	210	20	57.18	-4.23	-4.43	50	GTAGTGGATTCCGAGACCTA
B1c	146	170	25	61.72	-4.60	-4.09	44	TCTCTGGTATTACCTCAGCTTTAGC

Fig. 12 LAMP primers for Early transcribed membrane protein 11.2

v) Gene :- Ring-infected erythrocyte surface antigen

Gene ID :- PF3D7_0102200

Sequence :-

ATGAGACCTTTTCATGCATATAGTTGGATTTTTTCTCAACAATATATGGATACAAAAAATGTTAAGGAAAAAAATCCCACCATATATTCATTTGATGATGAAGAAAAAAGAAAT
GAAAATAAGAGCTTTTTAAAGGTGTTGTGTTCTAAACGTGGTGTTCTTCCAATTATTGGAAATACTATATATCATTTTAAATGGTAATCTTGGATATAATGGAAGTTCATCTTCTG
GCGTACAATTTACTGATAGATGTTCAAGAAATTTATACGGGGAAACATTGCCAGTAAACCCATATGCTGATTCTGAAAACCCAATAGTTGTAAGTCAGGTATTTGGTTTACCC
TTCGAAAAACCTACGTTTACCTTAGAAAGTCCTCCTGATATTGATCATACAAATATTTTGGGTTTTAATGAGAAGTTCATGACTGATGTAAATAGATATCGATATTCTAATAACT
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AAAATGCAAACTTTATGGGATGAAATAATGGATATTAATAAAAGAAAATATGATTCTTTAAAAGAAAAATTACAGAAAACTTACAGTCAGTACAAGGTTCAATATGATATGCC
AAAAGAAGCATATGAGAGCAAATGGACACAATGCATAAAACTTATTGATCAAGGAGGAGAGAACCTTGAAGAAAGATTGAACTCACAATTTAAAAACTGGTACAGGCAGA
AATATTTAAATCTTGAAGAATATAGAAGATTGACTGTGTTGAACCAAATCGCTTGGAAAGCTTTATCCAACCAAATTCAATATTCATGCAGAAAAATTATGAATAGTGACATTT
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AAAAGTGAACATTTATTAAAATTTATGGAACAATATCAAAAAGAAAGAGAAGCACATGTATCTGAATATTTATTAAATATATTACAACCATGTATAGCTGGTGATTCAAAATGG
AATGTACCAATTATAACAAAACTTGAAGGTTTAAAAGGATCTCGCTTTGATATACCAATATTAGAATCTTTAAGATGGATATTCAAACATGTCGCTAAAACACATTTGAAAAAA
TCCTCAAAATCAGCTAAGAAACTTCAACAGAGAACCCAGGCTAATAAACAAGAATTAGCAAATATAAATAATAACCTAATGAGTACATTGAAAGAATATGTAGGAAGTAGTG
AACAAATGAATTCAATAACATACAATTTCGAAAACATCAATTCCAATGTTGATAACGGAAACCAATCAAAAAATATTTCAGATTTAAGTTATACAGATCAGAAGGAAATATTA
GAAAAAATTGTTAGTTATATAGTAGATATTTCCCTTTATGATATAGAGAACACAGCTTTAAATGCCGCTGAACAATTGTTGTCAGATAATTCAGTAGATGAAAAAACTCTTAAA
AAGAGAGCTCAATCATTAAAAAAATTATCATCCATTATGGAGAGATATGCAGGTGGTAAAAGAAACGATAAAAAAGCAAAAAAATATGATACCCAAGATGTTGTAGGATATA
TTATGCATGGAATTAGCACAATTAATAAAGAAATGAAAAACCAAAATGAAAATGTACCCGAACATGTACAACATAATGCTGAAGCAAATGTAGAACATGATGCTGAAGAAA
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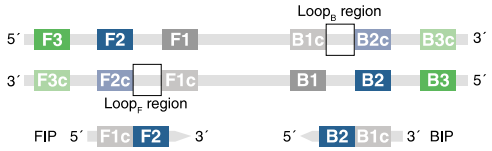
AATAA

Target: PF3D7_0102200, subsequence 1500-2700

Selected Primer Sets

Required primers:
F3
B3
FIP - Forward Internal Primer
BIP - Backward Internal Primer
LF - Loop Forward Primer
LB - Loop Backward Primer



SetID: PF3D7_0102200_P1

	5′ pos	3′ pos	len	Tm	5′ dG	3′ dG	% GC	(required oligos are orange)
F3	24	42	19	55.58	-3.83	-6.48	42	AACAAACTTCAGAAGCAGC
B3	242	266	25	56.27	-3.13	-4.74	32	CATATCCGTATTTATTGTACCATCT
FIP			45					TATCAGCATTAACACCAACACCTAATCCAACAATTGAAATCCCCG
BIP			45					ACTATCCATACCAAAGATCAGGTTCAAACTTGGTAGGCTTCGTTG
LF								
LB								
F2	43	62	20	57.97	-5.02	-6.98	45	TCCAACAATTGAAATCCCCG
F1c	83	107	25	60.35	-3.48	-4.08	36	TATCAGCATTAACACCAACACCTAA
B2	202	221	20	57.82	-3.66	-5.35	45	AAACTTGGTAGGCTTCGTTG
B1c	150	174	25	60.00	-3.47	-4.85	40	ACTATCCATACCAAAGATCAGGTTC

Fig. 13 LAMP primers for Ring-infected erythrocyte surface antigen

3.4) Reverse Transcriptase purification

The reverse transcriptase used for RT-LAMP reactions is Reverse Transcription Xeneopolymerase (RTx). We received this plasmid from Addgene. We Purified the enzyme using the protocol given in the methods section (2.3). We quantified the enzyme concentration using BSA standards, by running an SDS-PAGE and coomassie staining. The estimates suggest that the amount of purified polymerase is in the range of 10-15 µg/µl (Fig. 14) with the protein band running at 87 kDa.

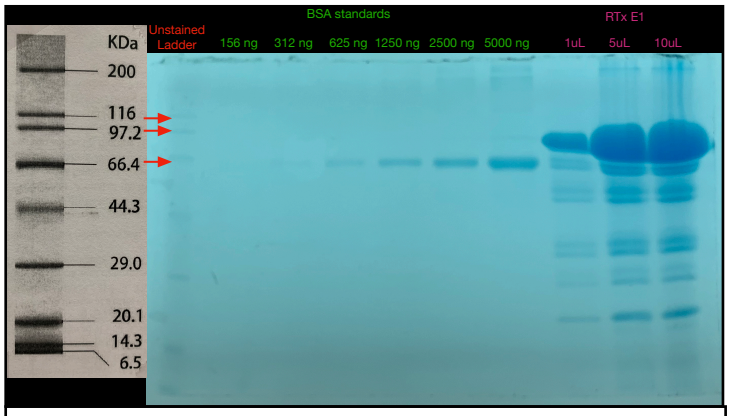


Fig. 14 Quantification of purified RTx polymerase

3.5) Reverse Transcription assays using RTx

The purified enzyme's activity is tested by synthesizing cDNA on isolated *plasmodium* RNA. The cDNA synthesized is subjected to PCR amplification using Taq polymerase and visualized by agarose gel electrophoresis (AGE). The protocols for Reverse transcription are given in the methods section (2.8). The readout for cDNA synthesis is PCR. In these assays, PCR is performed using Taq polymerase and primers for tRNA serine synthetase with 95 bp amplicon. The annealing temperature for Taq PCR is 57 °C. Amplicons are visualized by agarose gel electrophoresis (AGE) (Fig.15). The control reverse transcription (RT) is performed using the Promega reverse transcription kit section (2.7).

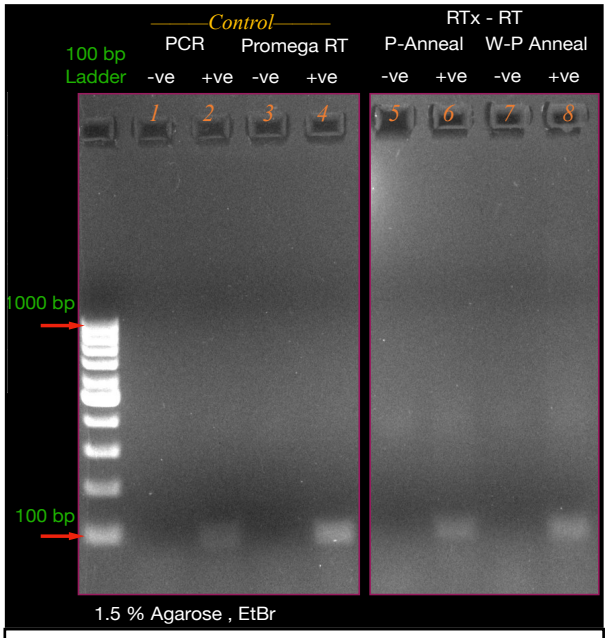


Fig. 15 AGE for cDNA synthesis using RTx polymerase

Control:-(*numbers represents wells in Fig. 15)
-[1] 'PCR -ve' well is negative PCR control where PCR without a template is performed. There is no band visible on AGE.
-[2] 'PCR +ve' well is positive PCR control where PCR is performed on template cDNA, amplicon band of 95 bp, is visible on AGE.
-[3] 'Promega RT -ve' well is a negative control for Reverse Transcription with RNA as a template and subjecting it to reverse transcription in the absence of reverse transcriptase (Promega RTase). No amplicon band is visible at the expected size.
-[4] 'Promega RT +ve' well is positive Reverse Transcription control with RNA template and performing reverse transcription using promega RT kit. This again gives a visible band at 95 bp amplicon after PCR.
Assays using RTx:- (*numbers represent wells in Fig. 15)
-[5] 'P-Anneal -ve' well is a negative assay in which no RTx was added during the reaction.
-[6] 'P-Anneal +ve' well is a positive assay in which RTx is added for cDNA synthesis, which also shows the band on the AGE after PCR as visible in '+ve PCR' and 'Promega RT +ve'.
-[7] 'WP-Anneal -ve' is a negative assay where RTx enzyme is not added to the reaction.
-[8] 'WP-Anneal +ve' in this well RTx is added for cDNA synthesis. On PCR 95 bp amplicon is again visible on AGE (Fig. 15).

'P-Anneal' and 'W-P Anneal' are two RNA treatment conditions with random hexamers. Random hexamers are 6 bp DNA primers. P-Anneal implies RNA was heated to 70 °C and rapidly chilled in an ice slurry in the presence of random hexamers. Whereas 'W-P Anneal' is the step in which no such treatment is involved and cDNA is synthesized directly in the reaction mix with random hexamers and RNA. The Primer annealing step is performed to trap primers in RNA's secondary structure.

3.6) Strand displacing polymerase purification

The LAMP reactions are performed using Geobacilllus Steareothermophilus polymerase 1 large fragment (Bst lf). We received the plasmid for this enzyme from Addgene. We purified it using the protocol given in the methods section (2.3). We next quantified the concentration of the protein using BSA standards, by SDS-PAGE and coomasie staining. The band for Bst lf runs at 67 kDa. On comparing loaded enzyme elutions, we estimate that the concentration is in the range of 5-10 µg/µl (Fig. 16).

Fig. 16 Quantification of purified Bst lf

3.7) LAMP assays using Bst lf

LAMP assays with purified Bst lf were performed on cDNA and PCR products for testing the enzymatic activity. cDNA is synthesized using Promega RT kit using random hexamers section (2.7). PCR amplicon used as the target template for Bst lf is synthesized using GAPDH primers (F3-B3) and cDNA of parasite.

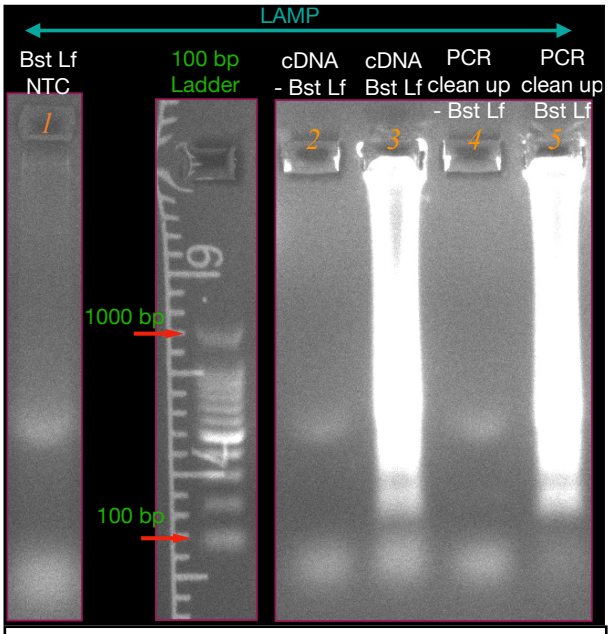


Fig. 17 LAMP reactions using Bst lf on cDNA and GAPDH PCR amplicons

(*numbering is to represent wells in Fig. 17)
-[1] 'Bst lf NTC' well in (Fig. 17) is a negative control where no template is added in the LAMP reaction. There are smudges visible at size lesser than 100 bp and at 500 bp as well. But the rest of the lane appears empty. Here the NTC stands for no template control.

-[2] 'cDNA -Bst lf' is a negative control with no Bst lf present in reaction to perform LAMP with cDNA as template. The lane looks identical to NTC control.

-[3] 'cDNA Bst lf' marked well has LAMP reaction performed on cDNA synthesized from parasites RNA using standard promega kit. The lane shows a bright illuminated smear, similar to ladder like profile which is characteristic of LAMP reactions.

-[4] 'PCR clean up -Bst lf' is again a negative assay with no Bst lf. GAPDH PCR amplicons was added to reaction mix as template. The lane is again empty with smudge marks similar to NTC.
-[5] 'PCR clean up Bst lf' shows AGE for LAMP assay performed using Bst lf on GAPDH PCR amplicons.

3.8) RT-LAMP assays with in house purified enzymes.

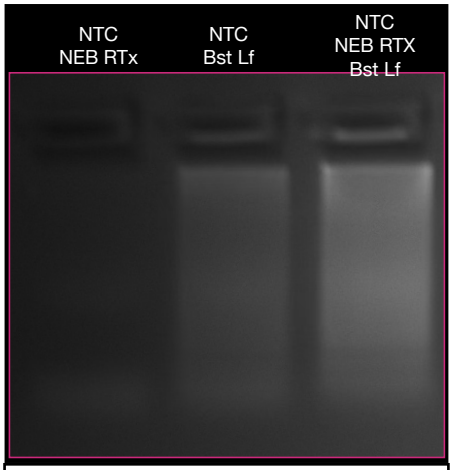


Fig. 18 No template control RT-LAMP reactions using Bst lf and NEB's RTx

With individual components working we started performing RT-LAMP reactions in one pot using in-house purified enzymes. But we were getting smear-like amplicons even in the absence of a template that is in no template control reaction. This showed that some amplification is happening without the target template present in the reaction vial. (Fig. 18) shows the AGE for NTC reactions. Here the RTx used is from NEB and Bst lf is in-house purified.
See (Fig. 18)-
-'NTC NEB Rtx' is a negative control which had all constituents for performing RT-LAMP reaction except for Bst lf and template RNA. The lane looks clear with no smear visible.
-The 'NTC Bst lf' lane is again a negative control with components of RT-LAMP reaction but without RTx and RNA.
-'NTC NEB RTx Bst lf' is a negative control in the absence of an RNA template to perform the RT-LAMP reaction. The image again shows that there is some kind of smear formation and is brighter, compared to negative control in 'NTC Bst lf'.

We tried circumventing the issue of false amplification, which is characteristic of LAMP reaction due to primer dimer formation and undergoing self-amplification, by changing reaction conditions like salt concentrations, temperature, primer ratio etc. But we were unsuccessful in our endeavours to completely eliminate spurious amplification. We next started using a commercial enzyme kit to perform RT-LAMP reactions.

3.9) RT-LAMP reaction for selecting the primer set

We are using NEB's RT-LAMP kit which has pre-formulated enzyme (RTx and Bst), buffer and dNTPs. This kit additionally needs primers and template RNA to carry out RT-LAMP reactions.

We screened for genes that can be used in our assays. (Fig. 19) shows AGE image for the assays

-[1] 'RTLAMP mix NFW' is negative control where only pre-formulated enzyme mix along with NFW is subjected to RT-LAMP reaction conditions section (2.10). The lane is clear with no signs of amplification.

-[2] 'RTLAMP mix RNA NFW' is again a negative control with no primers but all other reagents involved in the RT-LAMP reaction. This again gave no amplification inferred from the absence of a smear like pattern in the lane.

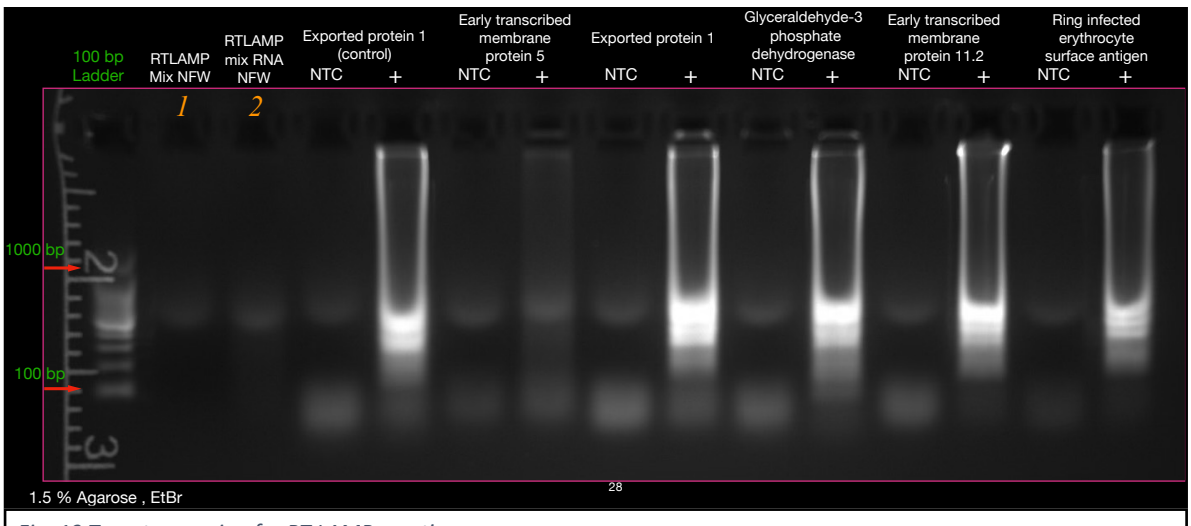


Fig. 19 Target screening for RT-LAMP reaction.

-Next, we started performing an RT-LAMP reaction with all the components (pre-formulated mix, primers, parasite RNA) together. We did this for individually selected targets. Each reaction had a specific LAMP primer set for a specific target gene. (Fig. 19) shows the profile for the RT-LAMP reactions. '+' marked lane is for RT-LAMP reaction with all components. 'NTC' is a negative control for verifying no spurious amplification takes place in the absence of RNA template. This is repeated for 5 selected genes. Additionally, one extra set of target primers marked (Exported Protein 1 control) is also added as a positive control. This set of primers is taken from published work for LAMP-based detection (Kemleu et al., 2016).

These assays show that all the designed primers work efficiently except for Early transcribed membrane protein 5. The rest of the designed primers give amplification smear similar to the control primers of Exported Protein 1.

We proceed forward with a primer designed for Exported Protein 1, GAPDH, Early transcribed membrane protein 11.2 and Ring infected erythrocyte surface antigen for sensitivity assays.

3.10) Sensitivity assays for RT-LAMP reaction

With the above selected primer sets we next set up sensitivity assays for RT-LAMP. We diluted purified parasite RNA in NFW serially to get detection ranges of our RT-LAMP assay. We started with 2 nano grams of parasite RNA and diluted it to 125 pico grams using nuclease-free water serially.

(Fig. 20) shows that all the selected primer sets were able to detect 125 pico grams of RNA molecules. The color coding is applied for primer sets in (Fig. 20), for identification of primer sets corresponding to AGE lanes.

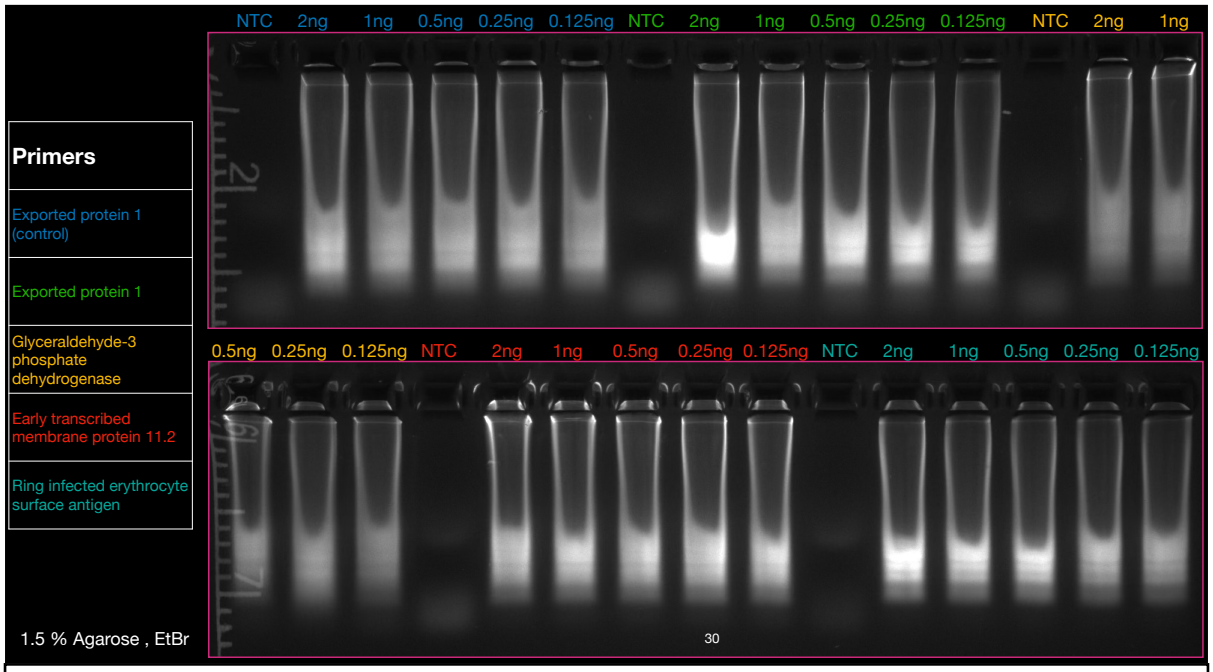


Fig. 20 Sensitivity assays for RT-LAMP reaction. NTC is a no template control reaction

3.11) Specificity assays for RT-LAMP reaction

The specificity assay for the RT-LAMP reaction was performed using RNA from fresh RBC lysate. We performed RT-LAMP reactions to screen out primer sets that show amplification with uninfected RBC RNA. (Fig. 21) shows AGE for the specificity assays for primer sets.

For each primer set targeting a gene, 2 RT-LAMP reactions are performed (Fig. 21). 'NTC' and '+', where 'NTC' reaction is a negative control for RT-LAMP reaction with no template in reaction and '+'

labelled well shows that the reaction is performed in presence of uninfected RBC's RNA. The '+' lane looks identical to the 'NTC' lane. Implying no amplification taking place in the presence of RBC RNA.The 5 RT-LAMP reactions are performed targeting the selected target genes using in-house designed primers. Color coding is maintained in (Fig. 21). Additionally one more primer set has been used for RT-LAMP reaction, 'Exported protein 1' primers which are already published as a positive control. A negative control was also set up without any primers set denoted by 'RTLAMP Mix RBC' in (Fig. 21). Here RBC RNA is mixed with the NEB RT-LAMP kit.

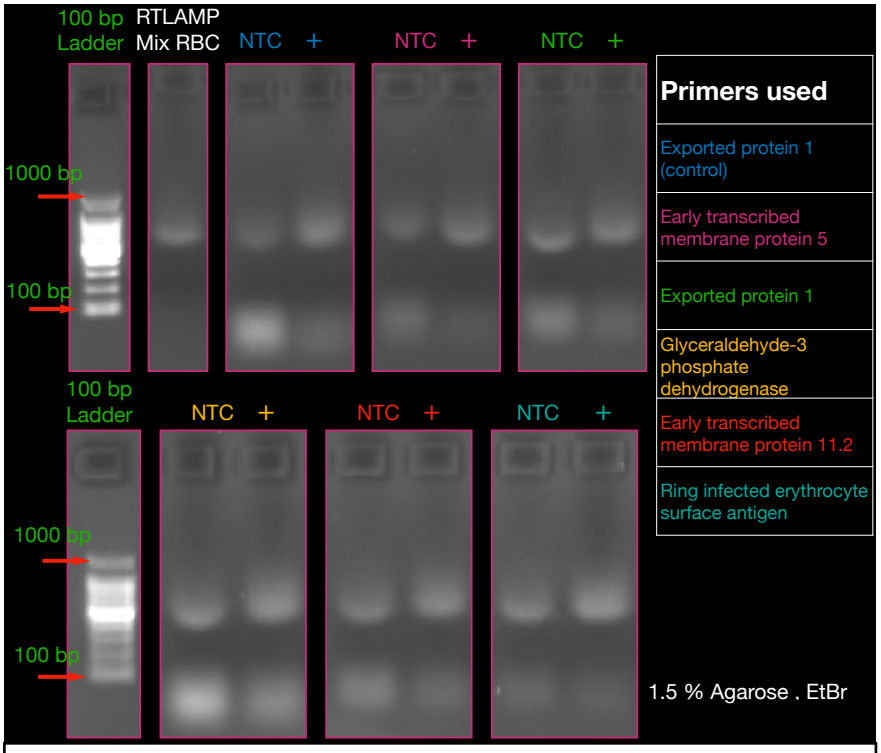


Fig. 21 RT-LAMP specificity assay with RBC RNA, targeting screened templates

3.12) RT-LAMP assays on infected RBC

Since our objective is to build a malaria detection kit which will be user-friendly we started experimenting with parasite detection in infected RBCs directly. This reduces the steps required for sample processing thus reducing time for detection.We begin with extracting RNA from infected RBC directly using the Trizol extraction section (2.6). (Fig. 22) is the AGE image for the assays. RT-LAMP was set up for 4 target genes screened in section (3.9) and additionally, a control primer set was used as a positive control for the assays.

(Fig. 22) is AGE for the RT-LAMP assay. The colour coding at the top shows the target primer sets used. 'NTC' is for no template control. 'RBC' is for uninfected RBC's RNA as a template. 'Pf' lane is for the parasite's RNA and '10% 'is infected RBC RNA which is isolated directly through Trizol extraction. The parasitemia in these infected RBCs was about 10% quantified microscopically by Giemsa staining. (Fig. 22) shows detection of parasite RNA is possible directly in infected RBCs

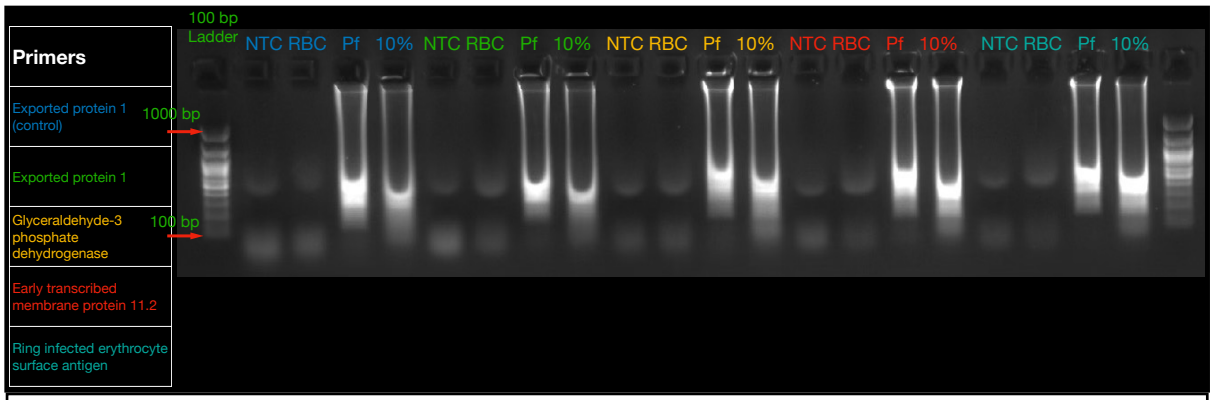


Fig. 22 RT-LAMP on 10% iRBC parasitemia

3.13) Sensitivity assays on infected RBC for parasite detection

We next tested the sensitivity of our RT-LAMP reactions by diluting the infected RBC with uninfected RBCs. All sets of primers used for the RT-LAMP reaction in section (3.12) are used for sensitivity assays here. The 10% RBC were diluted to 1%, 0.1% and 0.01% parasitemia by adding uninfected RBCs. RNA is purified directly from the RBC sample and used for RT-LAMP assays. Fig. 23 shows the AGE for the RT-LAMP assays for the dilution of 10% parasitemia blood.

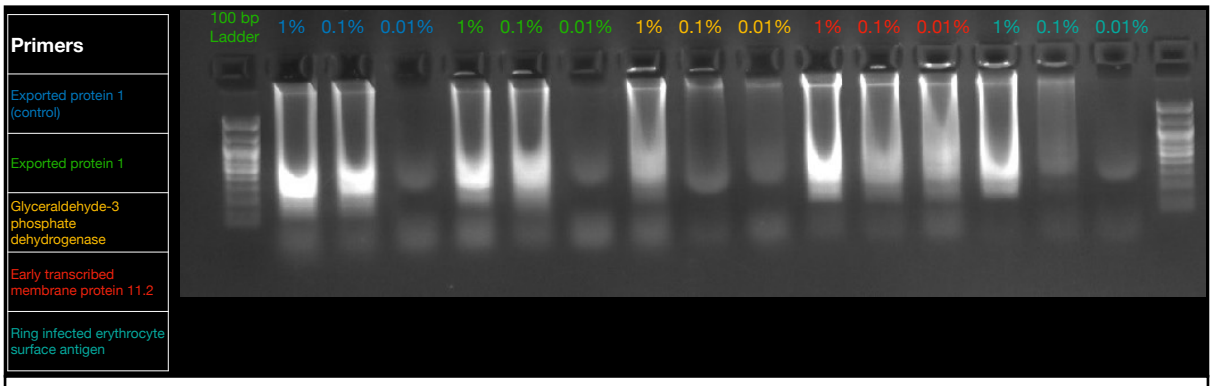


Fig. 23 RT-LAMP on 1% , 0.1% and 0.01% iRBC parasitemia

For each primer set 1%, 0.1%, and 0.01% parasitemia-infected blood is used. The RNA isolated from the lysis of infected RBC is a template for our reaction. The (Fig. 23) AGE shows that all the primer sets can detect parasite RNA at 0.1% parasitemia. Primer set targeting Early transcribed membrane protein 11.2 (ETRAMP 11.2) can detect parasite RNA even at 0.01% parasitemia. Even the control primer sets failed to amplify parasite RNA at 0.01% parasitemia.

Next, we tested for the sensitivity of ETRAMP 11.2 primer sets. We started with 2% parasitemia-infected RBCs and serially diluted them from 2% to 0.000002% by serially diluting infected RBCs with uninfected RBCs and isolating RNA directly using trizol extraction. (Fig. 24) shows the AGE. Smear is visible in all dilution till. Implying detection is possible at such dilutions as well. In 1 µl of blood, there are approximately $5.6*10^6$ RBCs so this detection range suggests that we can detect up to 0.1 parasite per microliter of blood.

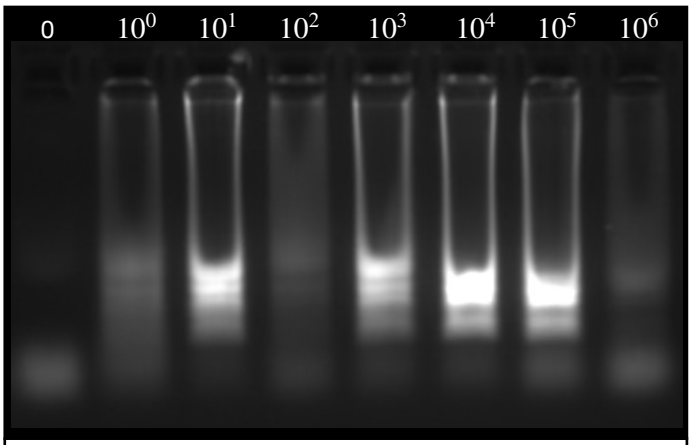


Fig. 24 RT-LAMP on dilutions of iRBCs from 2% to 0.000002%. The power of 10 denote dilutions factor.

3.14) Turbidity visualisation

RT-LAMP reaction produces huge amounts of pyrophosphates during the reaction thus increasing turbidity in the reaction vial. (Fig. 25) shows turbidity of RT-LAMP with 125 pg RNA, in contrast to the NTC RT-LAMP reaction denoted by the '-' which is relatively transparent.

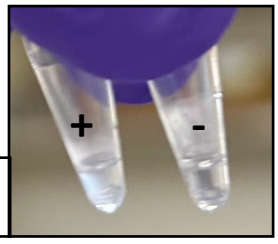


Fig. 25 Turbidity in RT-LAMP reaction. '+' represents reaction with 125 pg parasite RNA and '-' is NTC control.

4.Discussion

We started with screening for P. falciparum RNA transcripts as a target for the detection of parasites. For this, we used RNA sequencing data from (Tonkin et al., 2018) they have sequenced parasite transcripts from different patient samples. We chose 5 patient samples with uncomplicated malaria, as these patients will serve as a good proxy for asymptomatic conditions. We then analyzed the RNA sequencing data and quantified the transcripts using FPKM values on Galaxy Servers as in methods section (2.1). We applied constraints viz. 1) Highest abundance, 2) Transcript length greater than 100 bp, 3) No similarity with human and other organisms' genome. By applying these constraints we were able to pre-screen a few transcripts given in (Table. 2). These constraints screened for transcripts based on high abundance which means detection of parasites with greater sensitivity is possible and since these transcripts were chosen to be unique to the parasite, detection will be specific. With the list of transcripts in hand to choose from we next started designing LAMP primers on them. We looked for random splicing events on the transcripts in IGV and avoided either the region on the transcript or the transcript as a whole which had high splicing events. This reduces the risk for primers to fail during amplification, negating the chances of primer-template sequence non-complementarity. We designed primers using NEB's LAMP primer designing tool as described in section (3.3) and we were able to design primers on 5 target genes (Table. 13).

Our objective was to develop an affordable, sensitive diagnostic kit. So we started with synthesizing enzymes required for the reaction in-house. This helps us bring down the cost of making a detection module and will reduce dependency on outsourcing. The 2 enzymes that we need for RT-LAMP reactions are reverse transcriptase for converting RNA to cDNA and a strand displacing DNA polymerase which performs LAMP reaction on cDNA. Here we choose Reverse Transcription Xenopolymerase (RTx) as our reverse transcriptase and Geobacilllus Steareothermophilus polymerase 1 large fragment (Bst lf) as strand displacing DNA polymerase. These two enzymes are chosen for their myriads of unique characteristics. RTx and Bst lf are compatible with RT-LAMP reactions as they have optimum activity in similar buffers and temperature ranges of 60-70 °C (Hoffmeisterová et al., 2022; Milligan et al., 2018). RTx polymerase is an extremely sensitive enzyme that detects even femto grams of RNA (Hoffmeisterová et al., 2022). It also has proofreading capabilities making it highly specific and accurate in its function (Ellefson et al., 2016). It can also process DNA as its substrate for polymerization. On the other hand, Bst lf is a strong strand displacing polymerase (Milligan et al., 2018). It is a highly processive enzyme with 4 times the processivity compared to Taq polymerase (Kiefer et al., 1997). These characteristics make these enzymes ideal workhorse for RT-LAMP reactions. We were able to successfully purify and demonstrate their activities, validating the purification protocol. However further optimizations are needed for making these enzymes work at diagnostic application accuracy. Standardization and optimizations are needed to reduce the spurious amplification profiles in the no-template reaction section (3.8), which is typical of LAMP reactions. This can be due to complex primer designs that loop out and complement each other. In the absence of proper reaction conditions, it is possible for the enzymes to self-amplify these primers on their own without the requirement of a template. This random amplification can be eliminated by using extremely pure primers as normal primer purification by desalting tends to have other byproducts. Secondly optimizing for reaction temperature for better binding and specificity with template. Optimizing for ratios of outer and inner primer to make the system work robustly and lastly by optimizing for the chemistry of the reaction like changing salt, and enzyme concentrations and adding additives like DMSO, betaine, and BSA can reduce the random amplification. We did these optimizations and reached a reaction scheme where no amplification is visible in NTC (Fig. 19) using NEB's RT-LAMP kit. However, for in-house purified enzymes challenge remains to be resolved.

We next shifted our attention to performing RT-LAMP reactions using a commercial kit. With this, we were able to identify working primer sets for the detection. We then moved on to, sensitivity assays using purified parasite RNA and were able to show detection even at 125 pico grams. (Fig. 20) Shows that our detection scheme is sensitive and even lower detection levels are yet achievable. We also performed specificity assays on RNA isolated from RBCs (Fig. 21), this shows that the reaction scheme is specific to parasites RNA. Our objective is to develop a user-friendly detection module. Thus we tried assays on RNA isolated directly from infected RBCs instead of isolating parasite first

and then extracting RNA. This will shorten the sample processing step and reduce user dependency for diagnosis. We did sensitivity assays on the infected RBC RNA in sections (3.12) and (3.13). Here we were able to show that at dilution of 0.000002% parasitemia, detection with our scheme is possible by targeting the ETRAMP 11.2 template (Fig. 24). In 1 microliter of human blood there are approximately $5.6*10^6$ RBCs. This implies our module is detecting 0.1-0.2 parasites per microliter of blood alternatively, 1-2 parasites in 10 microliter of blood. This indicates that we can detect parasites at sub-microscopic densities. The sensitivities established in our assay schemes are sufficient enough for screening out asymptomatic cases effectively. Further, we also show that visualization of turbidity is enough to distinguish between positive and negative samples at extremely low concentrations of parasite's RNA (Fig. 25).

To further simplify diagnosis and make detection more robust. We are exploring a CRISPR-Cas-based detection module using an LFA strip. For which we have already laid out a scheme. We will be using the BrCas12b enzyme (Nguyen et al., 2021). This enzyme is compatible with working under the RT-LAMP reaction condition and it will be used for targeting RT-LAMP amplicons as explained in the introduction section. We have obtained the tracrRNA sequence for this enzyme from (Nguyen et al., 2021). We will be performing an in-vitro transcription reaction to synthesize specific sgRNAs, targeting RT-LAMP amplicons at specific sites. Using modified oligomers and LFA strips we will be simplifying readout for detection. Observing the striping patterns on the strip will be enough to verify the infection status (Fig. 6). Further we are simplifying the sample processing step. Which will require brief lysis of iRBCs releasing parasite's RNA and proceeding with RT-LAMP assay. These advancements will make our detection module user-friendly, and reduce the dependency on the operator. (Fig. 7) is a schematic for our complete detection module.

Further sensitivity can be increased by screening for transcripts that are present in higher abundance, which we did not consider here for example rRNA due to the unavailability of data sets. It is also possible to devise this reaction scheme for the detection of different species of *plasmodium* by choosing a common transcript amongst different species. We also need to validate RT-LAMP reaction on whole blood lysate for its clinical validation.

To conclude we were able to successfully demonstrate that the RT-LAMP reaction scheme developed here is capable of detecting infected RBCs with extreme sensitivity. With detection ranges up to single parasite levels.

A brief schematic for RT-LAMP reaction is given in Fig. 26. RT-LAMP reaction needs 4 primary components to be carried out.

1. RNA template
2. LAMP primers
3. Reverse Transcriptase
4. Strand displacing DNA polymerase

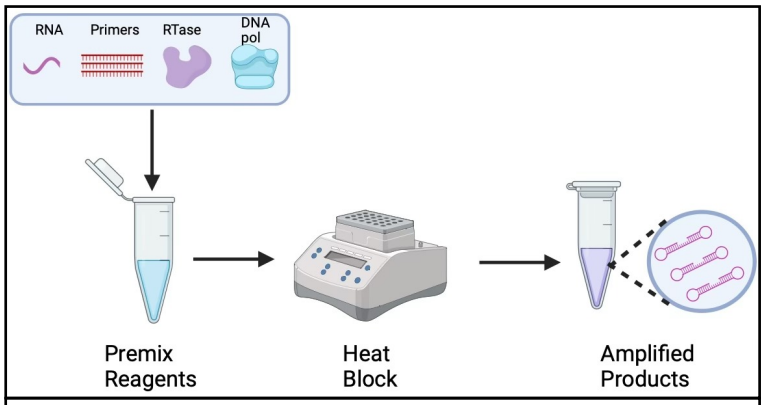


Fig. 26 RT-LAMP reaction scheme

These reagents are mixed with reaction buffer and dNTPs to conduct an RT-LAMP reaction by incubating at a single temperature (here 64 °C) for 40 minutes. These initial results generated have shown that our module can be a major stakeholder in malaria eradication efforts if developed further. Mass screening at remote places under resource constraints is possible. This kind of module once devised can be easily modified to diagnose another infectious disease. Thus making diagnostics Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end-users (ASSURED) (Ali et al., 2020).

References

1. Ali, Z., Aman, R., Mahas, A., Rao, G. S., Tehseen, M., Marsic, T., Salunke, R., Subudhi, A. K., Hala, S. M., Hamdan, S. M., Pain, A., Alofi, F. S., Alsomali, A., Hashem, A. M., Khogeer, A., Almontashiri, N. A. M., Abedalthagafi, M., Hassan, N., & Mahfouz, M. M. (2020). iSCAN: An RT-LAMP-coupled CRISPR-Cas12 module for rapid, sensitive detection of SARS-CoV-2. *Virus research*, *288*, 198129. https://doi.org/10.1016/j.virusres.2020.198129

2. Bhadra, S., Maranhao, A. C., Paik, I. and Ellington, A. D. (2021). A One-enzyme RT-qPCR Assay for SARS-CoV-2, and Procedures for Reagent Production. Bio-protocol 11(2): e3898. DOI: 10.21769/BioProtoc.3898.

3. Britton, S., Cheng, Q., & McCarthy, J. S. (2016). Novel molecular diagnostic tools for malaria elimination: a review of options from the point of view of high-throughput and applicability in resource limited settings. Malaria journal, 15, 88. https://doi.org/10.1186/s12936-016-1158-0

4. Cowman, A. F., Healer, J., Marapana, D., & Marsh, K. (2016). Malaria: Biology and Disease. Cell, 167(3), 610–624. https://doi.org/10.1016/j.cell.2016.07.055

5. Crutcher JM, Hoffman SL. Malaria. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 83. A

6. Das, A., Anvikar, A. R., Cator, L. J., Dhiman, R. C., Eapen, A., Mishra, N., Nagpal, B. N., Nanda, N., Raghavendra, K., Read, A. F., Sharma, S. K., Singh, O. P., Singh, V., Sinnis, P., Srivastava, H. C., Sullivan, S. A., Sutton, P. L., Thomas, M. B., Carlton, J. M., & Valecha, N. (2012). Malaria in India: the center for the study of complex malaria in India. Acta tropica, 121(3), 267–273. https://doi.org/10.1016/j.actatropica.2011.11.008

7. Dave, B., Kanyal, A., Mamatharani, D. V., & Karmodiya, K. (2022). Pervasive sequence-level variation in the transcriptome of Plasmodium falciparum. NAR genomics and bioinformatics, 4(2), lqac036. https://doi.org/10.1093/nargab/lqac036

8. Ellefson, J. W., Gollihar, J., Shroff, R., Shivram, H., Iyer, V. R., & Ellington, A. D. (2016). Synthetic evolutionary origin of a proofreading reverse transcriptase. Science (New York, N.Y.), 352(6293), 1590–1593. https://doi.org/10.1126/science.aaf5409Fola,

9. Fola, A. A., Feleke, S. M., Mohammed, H., Brhane, B. G., Hennelly, C. M., Assefa, A., Crudal, R. M., Reichert, E., Juliano, J. J., Cunningham, J., Mamo, H., Solomon, H., Tasew, G., Petros, B., Parr, J. B., & Bailey, J. A. (2023). Plasmodium falciparum resistant to artemisinin and diagnostics have emerged in Ethiopia. Nature microbiology, 8(10), 1911–1919. https://doi.org/10.1038/s41564-023-01461-4

10. Gunda, R., Chimbari, M. J., & Mukaratirwa, S. (2016). Assessment of Burden of Malaria in Gwanda District, Zimbabwe, Using the Disability Adjusted Life Years. International journal of environmental research and public health, 13(2), 244. https://doi.org/10.3390/ijerph13020244

11. Hoffmeisterová, H., Kratochvílová, K., Čeřovská, N., Slavíková, L., Dušek, J., Muller, K., Fousek, J., Plchová, H., Navrátil, O., Kundu, J. K., & Moravec, T. (2022). One-Enzyme RTX-PCR for the Detection of RNA Viruses from Multiple Virus Genera and Crop Plants. Viruses, 14(2), 298. https://doi.org/10.3390/v14020298

12. https://lamp.neb.com/#!/ NEB's LAMP primer design tool accessed. Accessed 10th January 2023

13. https://www.biorender.com/ Figure illustration made on Biorender. Accessed 15th February 2023

14. https://www.cdc.gov/parasites/malaria/ Malaria health report and epidemiology data

15. https://www.un.org/sustainabledevelopment/health/ UN SDGs for tropical & infectious diseases

16.Kemleu, S., Guelig, D., Eboumbou Moukoko, C., Essangui, E., Diesburg, S., Mouliom, A., Melingui, B., Manga, J., Donkeu, C., Epote, A., Texier, G., LaBarre, P., Burton, R., & Ayong, L. (2016). A Field-Tailored Reverse Transcription Loop-Mediated Isothermal Assay for High Sensitivity Detection of Plasmodium falciparum Infections. *PloS one*, *11*(11), e0165506. https://doi.org/10.1371/journal.pone.0165506

17.Kiefer, J. R., Mao, C., Hansen, C. J., Basehore, S. L., Hogrefe, H. H., Braman, J. C., & Beese, L. S. (1997). Crystal structure of a thermostable Bacillus DNA polymerase I large fragment at 2.1 A resolution. *Structure (London, England : 1993)*, *5*(1), 95–108. https://doi.org/10.1016/s0969-2126(97)00169-x

18.Leski, T. A., Taitt, C. R., Swaray, A. G., Bangura, U., Reynolds, N. D., Holtz, A., Yasuda, C., Lahai, J., Lamin, J. M., Baio, V., Jacobsen, K. H., Ansumana, R., & Stenger, D. A. (2020). Use of real-time multiplex PCR, malaria rapid diagnostic test and microscopy to investigate the prevalence of Plasmodium species among febrile hospital patients in Sierra Leone. *Malaria journal*, *19*(1), 84. https://doi.org/10.1186/s12936-020-03163-2

19.Milligan, J. N., Shroff, R., Garry, D. J., & Ellington, A. D. (2018). Evolution of a Thermophilic Strand-Displacing Polymerase Using High-Temperature Isothermal Compartmentalized Self-Replication. *Biochemistry*, *57*(31), 4607–4619. https://doi.org/10.1021/acs.biochem.8b00200

20.Moody A. (2002). Rapid diagnostic tests for malaria parasites. *Clinical microbiology reviews*, *15*(1), 66–78. https://doi.org/10.1128/CMR.15.1.66-78.2002

21.Nguyen, L. T., Macaluso, N. C., Pizzano, B. L. M., Cash, M. N., Spacek, J., Karasek, J., Miller, M. R., Lednicky, J. A., Dinglasan, R. R., Salemi, M., & Jain, P. K. (2022). A thermostable Cas12b from Brevibacillus leverages one-pot discrimination of SARS-CoV-2 variants of concern. *EBioMedicine*, *77*, 103926. https://doi.org/10.1016/j.ebiom.2022.103926

22.Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids research*, *28*(12), E63. https://doi.org/10.1093/nar/28.12.e63

23.Plucinski, M., Aidoo, M., & Rogier, E. (2021). Laboratory Detection of Malaria Antigens: a Strong Tool for Malaria Research, Diagnosis, and Epidemiology. *Clinical microbiology reviews*, *34*(3), e0025020. https://doi.org/10.1128/CMR.00250-20

24.Radfar, A., Méndez, D., Moneriz, C., Linares, M., Marín-García, P., Puyet, A., Diez, A., & Bautista, J. M. (2009). Synchronous culture of Plasmodium falciparum at high parasitemia levels. *Nature protocols*, *4*(12), 1899–1915. https://doi.org/10.1038/nprot.2009.198

25.Sato S. (2021). Plasmodium-a brief introduction to the parasites causing human malaria and their basic biology. *Journal of physiological anthropology*, *40*(1), 1. https://doi.org/10.1186/s40101-020-00251-9

26.Shirato K. (2019). Detecting amplicons of loop-mediated isothermal amplification. *Microbiology and immunology*, *63*(10), 407–412. https://doi.org/10.1111/1348-0421.12734

27.Tangpukdee, N., Duangdee, C., Wilairatana, P., & Krudsood, S. (2009). Malaria diagnosis: a brief review. *The Korean journal of parasitology*, *47*(2), 93–102. https://doi.org/10.3347/kjp.2009.47.2.93

28.Nguyen, L. T., Macaluso, N. C., Pizzano, B. L. M., Cash, M. N., Spacek, J., Karasek, J., Miller, M. R., Lednicky, J. A., Dinglasan, R. R., Salemi, M., & Jain, P. K. (2022). A thermostable Cas12b from Brevibacillus leverages one-pot discrimination of SARS-CoV-2 variants of concern. *EBioMedicine*, *77*, 103926. https://doi.org/10.1016/j.ebiom.2022.103926

29.Tian, Y., Liu, R. R., Xian, W. D., Xiong, M., Xiao, M., & Li, W. J. (2020). A novel thermal Cas12b from a hot spring bacterium with high target mismatch tolerance and robust DNA cleavage efficiency. *International journal of biological macromolecules*, *147*, 376–384. https://doi.org/10.1016/j.ijbiomac.2020.01.079

30.Tonkin-Hill, G. Q., Trianty, L., Noviyanti, R., Nguyen, H. H. T., Sebayang, B. F., Lampah, D. A., Marfurt, J., Cobbold, S. A., Rambhatla, J. S., McConville, M. J., Rogerson, S. J., Brown, G. V., Day, K. P., Price, R. N., Anstey, N. M., Papenfuss, A. T., & Duffy, M. F. (2018). The Plasmodium falciparum transcriptome in severe malaria reveals altered expression of genes involved in important processes including surface antigen-encoding var genes. *PLoS biology*, *16*(3), e2004328. https://doi.org/10.1371/journal.pbio.2004328

31.Waite, J. L., Suh, E., Lynch, P. A., & Thomas, M. B. (2019). Exploring the lower thermal limits for development of the human malaria parasite, Plasmodium falciparum. *Biology letters*, *15*(6), 20190275. https://doi.org/10.1098/rsbl.2019.0275

32.Zhao, Y., Chen, F., Li, Q., Wang, L., & Fan, C. (2015). Isothermal Amplification of Nucleic Acids. *Chemical reviews*, *115*(22), 12491–12545. https://doi.org/10.1021/acs.chemrev.5b00428