The optimization of loop-mediated isothermal amplification (LAMP) as a diagnostic tool for low-density, asymptomatic malaria infections

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Abstract

Current diagnostic tools for malaria lack the sensitivity to identify individuals with low-density infections. Asymptomatic low-density infections are common in malaria endemic regions and these individuals provide an important reservoir of infection that enables transmission to mosquitoes. Failure to detect these individuals threatens the global health goal of malaria elimination. Loop-mediated isothermal amplification (LAMP) is a technique to amplify DNA and has the potential to diagnose these individuals.

The LAMP assay was assessed in a field study in rural Vietnam. 5421 samples were collected and tested with a commercially available LAMP assay in Commune Health Care Centres in Binh Phuoc and Dak Nong Provinces. 101 positive LAMP cases (asymptomatic, smear, and RDT negative) were identified, with the proportion of positives ranging from 0.18% and 3.25% across five communes.

In order for LAMP to be used as a screening tool, it must be cost effective and have a workflow suitable for minimally trained end users. To achieve this, an in-house LAMP assay was developed and compared to PCR. The assay was combined with instrument detection to simplify decision making for the end user and improve sensitivity. The in-house assay was as sensitive as the PCR assay and cost US$0.60 per reaction compared to US$3.57 for PCR and US$8.23 for the commercial LAMP.

An integrated single cartridge, called T1, was assessed to further simplify this workflow of sample preparation, LAMP amplification and detection. Further development of the cartridge and the assay will be required for future deployment.

The LAMP assay is suitable for detection of low density infections in asymptomatically infected individuals in field settings and has the potential for cost effective population based screening.

Statement of authorship

Except where explicit reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma. No other person's work has been relied upon or used without due acknowledgment in the main text and bibliography of the thesis. No editorial assistance has been received in the production of the thesis without due acknowledgement. Except where duly referred to, the thesis does not include material with copyright provisions or requiring copyright approvals.

Leanna D Surrao
30 Aug 2019

Preface

The majority of work presented in this thesis was performed at the Burnet Institute in Melbourne, Australia, in the laboratory of Associate Professor Jack Richards. Part of this work was also performed in Vietnam, as part of activities run by the National Malaria Control Program and the National Institute for Malariology, Parasitology, and Entomology.

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List of publications

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Abbreviations

ACT	Artemisinin-based combination therapy
ASSURED	Affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users
B3	Backward primer
BIP	Backward inner primer
CDC	Centres for Disease Control and Prevention
DBS	Dried blood spots
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
F3	Forward primer
FIND	Foundation for Innovative New Diagnostics
FIP	Forward inner primer
FP	Forward probe
FSAT	Focused screening and treatment
G/C	Guanine/cysteine
G6PD	Glucose-6-phosphate dehydrogenase
GIS	Geographic information system
GPS	Global positioning system
HRP2	Histidine rich protein 2
IRS	Indoor residual spraying
ITN	Insecticide-treated net
LAMP	Loop-mediated isothermal amplification
LB	Backward loop primer
LCD	Liquid crystal display
LDH	Lactate dehydrogenase
LED	Light-emitting diode
LF	Forward loop primer

LLIN	Lon lasting insecticide-treated net
LOD	Limit of detection
MDA	Mass drug administration
mDNA	Mitochondrial deoxyribonucleic acid
MSAT	Mass screening and treatment
NAAT	Nucleic acid amplification technique
NFW	Nuclease free water
NIMPE	National Institute for Malariology Parasitology Entomology
NMCP	National Malaria Control Program
PCR	Polymerase chain reaction
POC	Point of care
qPCR	Real-time or quantitative PCR
RAI	Regional Artemisinin-resistance Initiative
RBC	Red blood cell
RNA	Ribonucleic acid
RP	Reverse probe
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
SOP	Standard operating procedure
ssDNA	Single stranded deoxyribonucleic acid
UV	Ultra violet
WHO	World Health Organization

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1 Literature review

1.1 Introduction to malaria

1.1.1 Burden of malaria worldwide

Malaria is a life-threatening, but preventable and curable disease, caused by the protozoan parasites of the *Plasmodium* genus. It is propagated by infected female *Anopheles* mosquitos. There are five species of parasite that commonly cause malaria in humans; *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (subspecies *curtisi* and *wallikeri*), and *P. knowlesi*. *P. falciparum* causes the greatest burden of mortality and morbidity globally [3]. The dominant species found in any particular area varies from region to region, but *P. falciparum* and *P. vivax* are generally the most common, except for pockets of *P. knowlesi*. Almost half of the world's population is at risk of malaria, with young children, pregnant women, and non-immune travellers being the most susceptible [3]. In 2017 there were an estimated 219 million cases worldwide (**Figure 1.1 A**). The WHO African Region had the largest burden with 200 million cases (92%), followed by the South-East Asia Region (5%) and the Eastern Mediterranean Region (2%) (**Figure 1.1 C**). Of those cases, there were 435,000 deaths worldwide (**Figure 1.1 B**), again with the largest burden being in the African Region (93%) and 266,000 (61%) of those were children under the age of 5 years old [1].

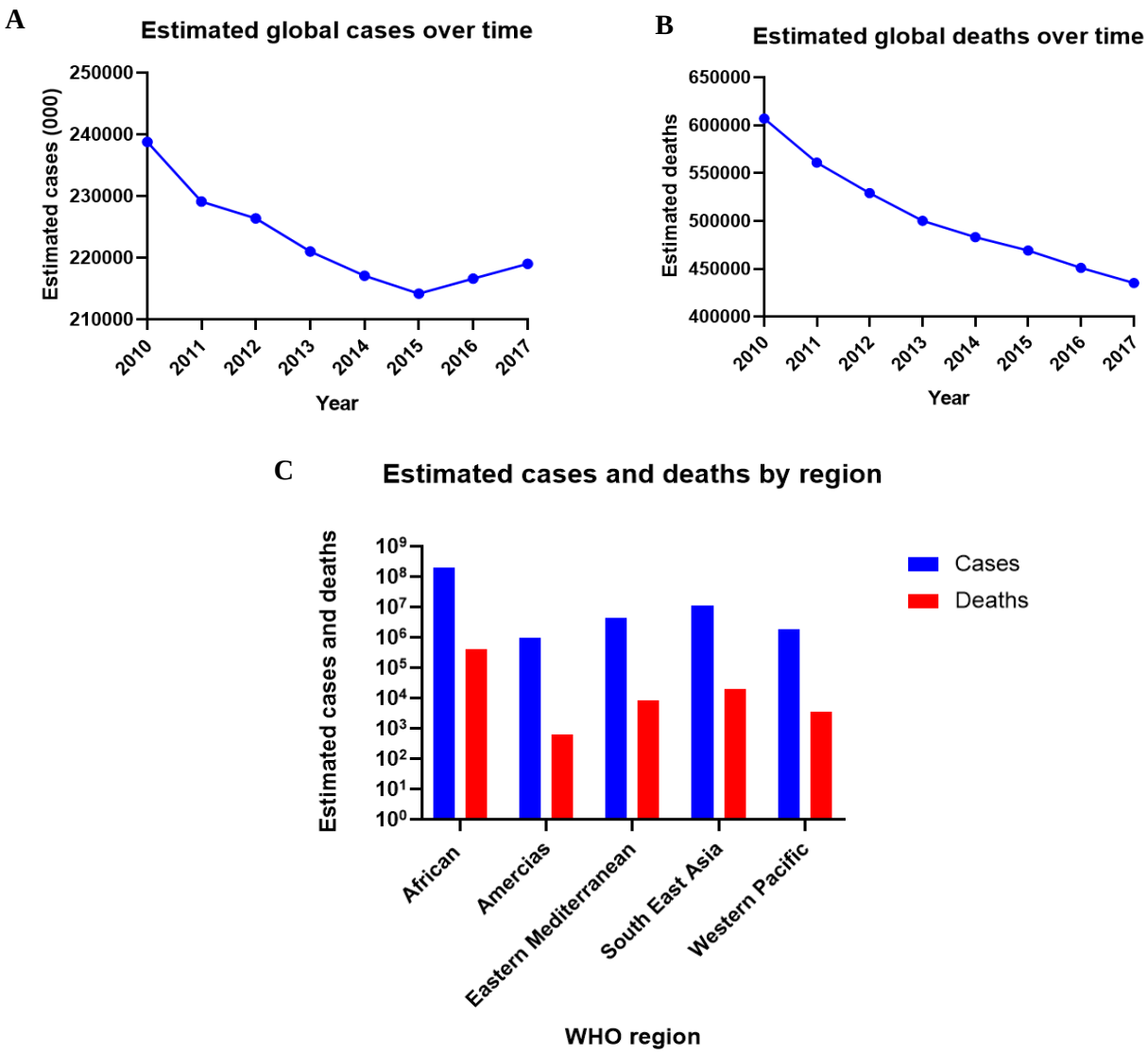

Figure 1.1: Prevalence of malaria cases and deaths worldwide and by region.
(A) Estimated malaria cases worldwide between 2010 and 2017, **(B)** reported global deaths during that same period, and **(C)** reported cases and deaths in 2017 stratified by WHO defined region [1].

The incidence of malaria remains high in some regions as demonstrated partly by the increase during the 2015-2017 period where no significant progress in reducing malaria was made (**Figure 1.1 A**) [1]. However, control and intervention have been very successful in other regions and in many countries becoming malaria free is an attainable goal. The Global Technical Strategy for Malaria 2016-2030, the framework to guide countries towards malaria elimination, sets the target of reducing incidence and mortality rates by at least 90% by 2030; with elimination in at least 30 countries in that timeframe (**Table 1.1**). This shift from disease control to elimination of malaria altogether in certain countries, places an emphasis on finding and treating all infected individuals.

Table 1.1: Goals outlined in the WHO Global Technical Strategy for Malaria 2016-2030

Goals	Milestones		Targets
	2020	**2025**	**2030**
1. Reduce malaria mortality rates globally compared with 2015	At least 40%	At least 75%	At least 90%
2. Reduce malaria case incidence globally compared with 2015	At least 40%	At least 75%	At least 90%
3. Eliminate malaria from countries in which malaria was transmitted in 2015	At least 10 countries	At least 20 countries	At least 35 countries
4. Prevent re-establishment of malaria in all countries that are malaria-free	Re-establishment prevented	Re-establishment prevented	Re-establishment prevented

1.1.2 *Plasmodium* parasite lifecycle

Transmission from infected mosquito to human occurs from sporozoite stage parasites contained in the saliva of the mosquitos (**Figure 1.2, Stage A**), entering into the cutaneous tissues of humans during mosquito feeding (**Figure 1.2, Stage B**). In this pre-erythrocytic cycle, sporozoites travel to the liver where they take-up residence in hepatocytes. In *P. vivax* and *P. ovale* infections, liver stage parasites can also develop into hypnozoites that can remain dormant for weeks, months, and even years after the initial infection [4]. Within the tissue schizont, the parasite under goes asexual replication to form thousands of daughter merozoites. Eventually, merozoites rupture out of the hepatocyte and re-enter the blood stream [3]. Here merozoites invade red blood cells (RBCs) and initiate a cycle of invasion, multiplication, rupture, and re-invasion of more RBCs. When merozoites invade RBCs they develop into either trophozoites or gametocytes.

Trophozoites mature and segment into schizonts which contain daughter merozoites. Schizonts then rupture RBCs releasing these merozoites and these merozoites then re-invade surrounding RBCs. This cycle leads to the increased parasitaemia associated with symptoms of clinical malaria (**Figure 1.2, Stage C**). The duration of this process varies between species from 24 (*P. knowlesi)*, 48 (*P. falciparum* and *P. vivax)*, 50 (*P. ovale*), and 72 hours (*P. malariae*).

Gametocytes undergo a sexual cycle of replication within the mosquito known as the sporogonic cycle [3]. Gametocytes are ingested when an uninfected mosquito takes a blood meal from an infected human (**Figure 1.2, Stage D)**. The male version of the gametocyte (microgametocytes) penetrate the female gametocytes (macrogametocytes) in the mosquito's stomach, generating zygotes. Zygotes mature into motile and elongated ookinetes which invade the gut wall and develop into oocytes. These oocysts grow, rupture, and release sporozoites which migrate to the salivary glands of the mosquito [3]. The cycle is perpetuated when these sporozoites are transmitted to another human during the next blood meal (**Figure 1.2, Stage A**).

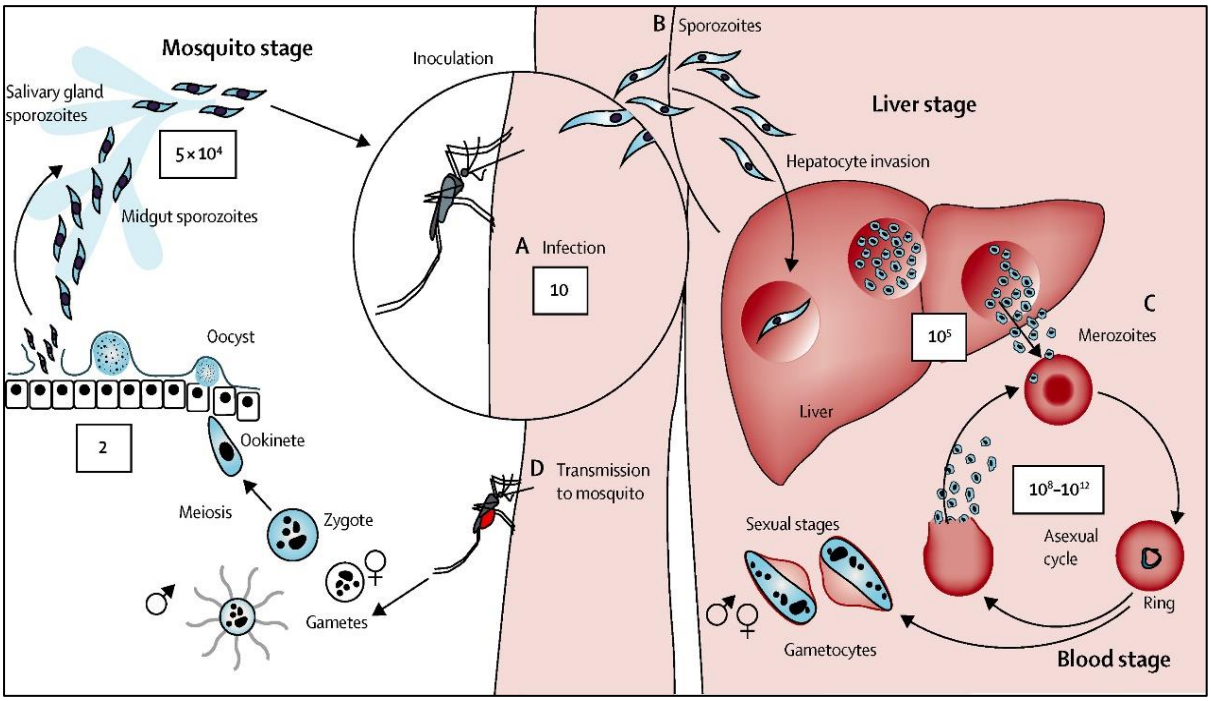

Figure 1.2: Plasmodium lifecycle.
The erythrocytic and sexual stages of the human and vector hosts of *Plasmodium* species (White et al. 2014).

1.1.3 Clinical manifestation and case management

Symptoms

The clinical manifestations of malaria are observed during the erythrocytic stage of the parasite life cycle (i.e. the pre-erythrocytic stage is asymptomatic). Patients may experience fever, nausea and vomiting, chills, and headaches in uncomplicated malaria cases [5]. Severe malaria occurs when infections are complicated by organ failure or when there are abnormalities of the blood or metabolism. This can manifest as cerebral malaria, severe anaemia, kidney failure, and other less common manifestations [5]. In endemic areas, individuals may develop naturally-acquired immunity after multiple infections. This immunity may afford protection from developing symptomatic malaria but is thought not to protect from infection itself. This immunity appears to wane over time without repeated exposure to parasites [6].

Treatment

The treatment of symptomatic malaria depends on the species of parasite, whether the case is uncomplicated or severe, and whether there is drug resistance in that area [5]. Determining the *Plasmodium* species causing the infection is important for clinical management and for public health purposes. Infections with some species, like *P. falciparum* and *P. knowlesi* can progress more quickly and are more likely to cause severe disease relative to other species of *Plasmodium*. In addition, some species like *P. vivax* and *P. ovale* may require treatment of both blood stage parasites and the hypnozoite form that remains dormant in hepatocytes. Furthermore, different species have different drug resistance patterns in different regions (e.g. artemisinin resistance for *P. falciparum*). It is therefore important for a diagnostic test to not only be able to detect a *Plasmodium* infection, but to also be able to differentiate between species [5].

For uncomplicated *P. falciparum* infections artemisinin-based combination therapies (ACTs) are recommended by WHO. ACTs usually consist of two or more active compounds, which have different modes of action and different half-lives. The artemisinin compound has a fast killing action, whereas the secondary compound is usually long-acting and present to clear any residual parasites. Primaquine

may also be administered as a gametocytocidal agent for *P. falciparum* infections in low-transmission areas, thus helping to reduce parasite transmission [7].

P. vivax infections are largely treated with chloroquine, although in some areas, where chloroquine-resistant *P. vivax* is prevalent, infections may be treated with ACTs. To ensure curative treatment of *P. vivax* (i.e. prevent relapse infections from hypnozoites), primaquine is ideally added to the treatment schedule. In any case where primaquine is administered, the glucose-6-phosphate dehydrogenase (G6PD) enzyme activity of the patient should be checked to prevent adverse side effects such as haemolysis. Primaquine should not be administered to pregnant women [7].

Severe malaria in adults is treated with injectable artesunate either into the muscle or blood stream for the first 24 hours. This is followed by an oral course of ACT once the patient is able to eat and drink normally to ensure the infection is completely cleared. Children under the age of six years old can be treated with artesunate suppositories if there is a delay accessing health care facilities for treatment.

1.1.4 Interventions against malaria

Preventative measures come in many forms; some that limit transmission by the vector and others that target the parasite directly. A long standing form of vector control has been to manage water bodies (particularly stagnant water) where mosquitos breed, through a combination of engineering and behavioural interventions. More recently traditional approaches have been complemented with modern forms of chemical control of vectors such as insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS). ITNs are physical barriers typically made of polyester that have been treated with insecticides such as permethrin, cyfluthrin, or alphacypermethrin [8] to protect people while they sleep. The introduction of long-lasting insecticide-treated nets (LLINs), which maintain their insecticidal activity for at least 3 years [9], resulted in steep declines in malaria cases where large amounts of the nets were distributed [9].

IRS involves spraying the interior walls of households in affected areas with insecticides similar to those used to treat ITNs. This protects household inhabitants of the household as mosquitos and other insects are killed when they come to rest on the sprayed surface. The effectiveness of IRS depends on

factors such as the spray coverage, the nature of the sprayed surface, temperature, and humidity [10]. Vector control has been an important contributor to malaria control efforts over the past decade.

Unlike ITNs and IRS, intermittent preventative therapy targets the parasite directly by using antimalarial drugs as intermittent doses in high risk populations. For example, sulfadoxine-pyrimethamine can be administered to pregnant women, infants, and children in high-transmission areas, regardless of whether they are infected or not (i.e. the emphasis is on intermittent administration of the drug without confirmatory testing, with the assumption that a large enough proportion of these populations will actually be infected and will therefore derive a benefit from the treatment). This can reduce malaria episodes, birth complications, and growth defects, as well as act as a prophylactic; [11] although the potential for the development of drug resistance should not be discounted.

Treatment regimes such as mass drug administration (MDA), mass screen and treat (MSAT), and focal screen and treat (FSAT) have also been used in malaria interventions. MDA involves the treatment of a population without prior diagnostic confirmation and irrespective of infection status. While it has seldom been used in malaria settings to date due to emerging drug resistance, the WHO's Malaria Policy Advisory Committee is now recommending MDA in specific situations such as when transmission is close to being interrupted or if imported infections are minimal [12]. In comparison, MSAT involves treating everyone in a population *after* they have been positively diagnosed. Although time and resource consuming, this method ensures that all positive infections identified are treated without risk of contributing to drug resistance due to over use of antimalarials [13]. Similarly to MSAT, FSAT interventions treat all active infections identified but rather than screening all individuals in a population, it focuses on screening at-risk individuals in proximity to confirmed malaria cases. This focused screening method conserves time and resources, but is dependent on the existence of spatial clustering of infections [14]. Each method can be implemented in a health system with varying levels of feasibility. To interrupt transmission, MDA must be conducted in a coordinated manner (often at repeated intervals), treatment must cover the majority of the population, and participants must adhere to the treatment regime even though they are not experiencing symptoms and haven't been tested. If this does not occur, transmission often returns to its original level, therefore it demands a high level of

community participation, and extensive and prolonged resource requirements from the health system. MSAT ensures only infected individuals are treated and collects valuable information on the epidemiology of malaria in a population, but is logistically challenging and resource intensive, making it difficult to implement sustainably in remote or resource limited settings. FSAT implementation is subject to many of the limitations of MSAT, with the addition of not treating an entire population simultaneously reducing impact on parasite reservoir. Highly sensitive diagnostic tests are also essential in both MSAT and FSAT intervention strategies as they are most suited to detecting cases in low transmission settings. The implementation requirements of MDA, MSAT, and FSAT may not be feasible in every setting, therefore future interventions should address these challenges; namely controlling for logistical challenges, time and resource burdens on health systems, acceptance from community, and ensuring the screening tool used is highly sensitive.

1.2 Malaria in the context of Vietnam

1.2.1 Current malaria status

Vietnam is a malaria endemic country but in past decades has made significant progress in its control efforts. Most of the country (67%) has low transmission status (0-1 case per 1000 population), or is malaria-free (26% of the county) with only 7% of the country having high transmission status (>1 case per 1000 population) [15]. In 2016, there were 4,161 reported cases and two reported deaths. All five species of *Plasmodium* are present but most cases are caused by *P. falciparum* (64%) and *P. vivax* (35%). Transmission in southern and central provinces is characterized by a seasonal peak between December and February, but transmission in the north is much more sporadic. The most common vectors throughout the country are *An. mimimus*, *An. dirus*, and *An. sundaicus* [15].

The health care system in Vietnam is established on a commune basis. Each commune has a designated health care centre in which trained staff manage all malaria related responsibilities such as diagnosis and treatment. Each commune is further divided into villages where Village Health Care Workers represent the community level malaria response such as health promotion and referral to Commune Health Care Centres. Government policy dictates that ITNs and LLINs are distributed free of charge

and that diagnostic testing and treatment are also cost free. Case reporting is mandatory in all sectors [15].

1.2.2 Control and elimination goals

There has been a substantial decline in reported cases of malaria (**Figure 1.3 A**) and deaths associated with malaria in Vietnam (**Figure 1.3 B**) between 2000 and 2010. This success has led 2020 targets stated in Vietnam's National Strategy for Malaria Control and Elimination, which are as follows; (1) morbidity below 0.15 per 1000 population, (2) mortality below 0.02 per 100 000 population, and (3) malaria eliminated in at least 40 communes. With more than 40 of 58 provinces now malaria free [15] Vietnam has achieved these targets.

In 2011, the Vietnamese Government launched the National Malaria Control and Elimination Programme with the goal of eliminating malaria by 2030. With much of the country currently malaria free, and the main burden of disease and transmission occurs in areas such as the central highlands and along country borders with Cambodia and Laos. The major challenges facing malaria elimination in this setting, such as forest malaria, population movements, and antimalarial resistance [16], will be further discussed in **Section 1.2.3**. The remote and forested areas where transmission continues, are home to many ethnic minorities still living by traditional means and conducting forest related activities such as logging. Given their location, they often have limited or no access to health care services despite having an increased risk of infection [17].

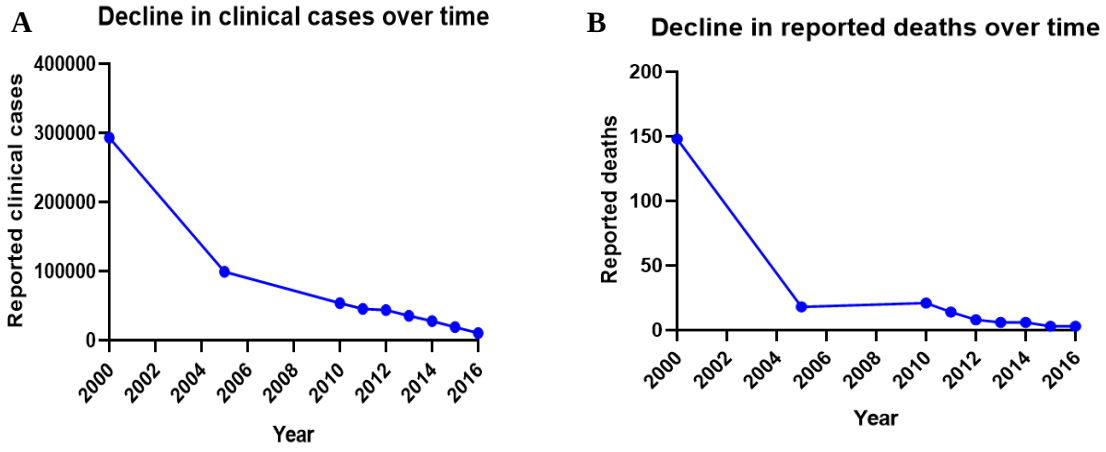

Figure 1.3: Decline of clinical cases and deaths in Vietnam over time.
A steady decline was seen in reported cases **(A)** and deaths **(B)** from 2010 to 2016 (WHO, 2018).

1.2.3 Challenges impacting malaria elimination progress

A major challenge for malaria elimination in the Greater Mekong Subregion is the largely uncontrolled cross-border movement of people, especially those living in forested areas. Many of these mobile and migrant populations, are at higher risk of infection and may pose the greatest transmission risk given their proximity to mosquitos during peak biting times. Their mobile nature means they are also often out of reach of health care services. Cross border movement between bordering countries like Cambodia and Vietnam is common as many people travel to sell produce or visit relatives.

A study by Gryseels and colleagues surveyed household heads and youths in villages along the Vietnam (n=401) and Cambodian (n=546) border to assess these border crossing patterns. Participant responses were categorized into the following sections; housing structure, sleeping patterns, cross-border mobility, and protection with bed nets. The study outlines some of the challenges faced in the region in the elimination of malaria.

Where residents live can directly increase their chances of being bitten by mosquitos. Houses in Cambodian villages were usually wooden stilted longhouses whereas homes in Vietnam were made of concrete without stilts. Many families had additional homes at their forest farms or rice fields in both countries, and in Vietnam these houses were similar to those in Cambodia (i.e. stilted bamboo or wooden huts). A similar study conducted in Sub-Saharan Africa found a 9-14% reduction in infection among participants living in houses made of finished material (e.g. metal) compared to those made with rudimentary materials (e.g. thatch) [18]. These open style houses offer little protection to residents during mosquito biting times.

Sleeping outside whether in the forest or in the village, also increases transmission risk. Many forest workers need to sleep in the forest during transmission season, which greatly increases their risk of getting bitten and contracting the parasite. Cambodian participants (51.4%) were more likely to engage in “deep forest activities” (e.g. hunting, logging, and fishing) than those in Vietnam (10.3%), while youth for both countries were similarly likely to sleep in hammocks outside as they became more independent from their parents (Cambodian youth=40.3% and Vietnamese youth=53%). Similarly to the open houses that some reside in, the activity of sleeping in the forest or simply sleeping outdoors

in the village poses a great risk. A study in Ghana also found that sleeping and engaging in other night time activities, increased the risk of getting bitten [19]. Activities such as night school, household chores, and moving livestock were observed during the peak biting times of the season in these populations. A reservoir of transmission will remain as long as mosquitos are able bite and infect humans.

Bed nets can be highly effective in controlling the vector, but only if insecticide treated and used appropriately. Bed net ownership and usage among adults was high in both Vietnam (100%) and in Cambodia (95.1%), but not all were insecticide treated or intact. Of the bed nets used in Vietnam 90.4% were insecticide treated and 89.8% were intact, compared to just 58.6% of bed nets being treated and 66.2% remaining intact in Cambodia. The importance of treated and intact bed nets was highlighted in a study in the Dominican Republic of Conga, which found a reduction in malaria prevalence after LLIN distribution campaigns, followed by an increase in prevalence the following year in communities that didn't receive new nets or weren't educated in their appropriate use [20].

As previously mentioned, it is common for people to cross borders into neighbouring countries. Such movement was more common in Cambodia than Vietnam with 46.3% of participants traveling to and spending the night in Vietnam, compared to 30% doing the reverse from Vietnam. Targeted surveillance and control in these border regions is of the utmost importance to capture those missed or out of reach of routine community screening.

1.3 Roadblocks to Elimination

1.3.1 Sub-microscopic reservoir

Elimination, defined as the interruption of local transmission [21], is attainable in Vietnam and other countries where control has been successful in recent years. When elimination is the focus, there is a need to detect all infections, even in asymptomatic people. Many people in these communities who are not experiencing any symptoms may still carry low levels of parasites and make up an important reservoir for ongoing parasite transmission [22, 23]. These low levels of parasitaemia can reflect chronic infections in immune individuals or inadequately treated current infections [24].

Low density infections are often undetectable by current diagnostics such as rapid diagnostic tests (RDTs) and light microscopy. A study by Thanh *et al* (2015) surveyed four villages (1450 individuals) in the forested area of Central Vietnam and found an asymptomatic reservoir of 22.6% using semi-nested multiplex polymerase chain reaction (PCR) compared to just 7.8% detected by microscopy. In another study of asymptomatic people in South West Vietnam, 3212 individuals were tested by PCR, RDT and microscopy. Detection was considerably higher using PCR (12%) compared to microscopy (4%) and RDT (3%) [25]. These studies clearly show that the level of asymptomatic infections is greatly underestimated when using traditional malaria diagnostics alone and more sensitive tests are needed to accurately measure this reservoir [22, 26].

In addition to identifying asymptomatic individuals carrying blood stage parasites, it is also important to detect those carrying the sexual stage of *Plasmodium*: the gametocyte. Gametocytes are infectious to mosquitos and therefore facilitate transmission between humans and mosquitos [27]. Identifying gametocytes is not only important in predicting the infectivity or transmission potential of an individual, but also to monitor parasite clearance by antimalarial drugs. Although gametocytes can be readily distinguishable from other life stages in some species, their density is often too low to be detected by light microscopy. Therefore, there is a need for more sensitive diagnostic methods, such as molecular approaches [28].

Test sensitivity is not the only reason current diagnostic tests are inadequate. The majority of malaria RDTs use an antigen capture approach. For most RDTs that detect *P. falciparum*, they capture the histidine rich protein 2 (HRP2) antigen. Recently there have been increasing reports of false negative RDTs due to *pfhrp2* gene mutations (resulting in the deletion of HRP2). Many cases of parasites lacking the *pfhrp2* gene have been identified in South America [29], more recently in India [30], Rwanda [31], Kenya [32] and Eritea [33]. Continuing to use RDTs in areas where HRP2 deletions are present, will further underestimate the true burden of infected individuals.

In all of the above scenarios, more sensitive and/or accurate diagnostic tests are required. New tests need to be feasible in resource limited settings, and cheap enough to be used in mass screening

interventions or incorporated into existing health care practices. Such characteristics will ensure that sufficiently large proportions of communities can be tested and treated to facilitate transmission.

1.3.2 *P. vivax* infections

P. vivax infections present unique challenges to both malaria control and elimination efforts. Almost half of the world's population is at risk of *P. vivax* infections, with Asian countries having the highest rates of endemicity [34]. In recent years there has been a shift in the species composition causing malaria cases, with an increase in the prevalence of *P. vivax* infections, such that it has surpassed that of *P. falciparum* in non-African countries [35]. This is largely due to the effectiveness of interventions against *P. falciparum* causing falling numbers of *P. falciparum* cases, while the prevalence of *P. vivax* has remained largely unchanged. *P. vivax* infections are unique in that: blood-stage infections are usually of a much lower parasite density than *P. falciparum* therefore making diagnostic testing more difficult, relapse is possible due to the presence of liver-stage hypnozoites, treatment can be complicated by the need for blood-stage and liver stage antimalarials, and transmission potential is high due to early production of gametocytes. Moreover, diagnosis with RDTs largely relies on parasite lactate dehydrogenase (LDH) because HRP2 is not present in *P. vivax*; however, LDH is less abundant in *P. vivax* than HRP2 is in *P. falciparum*.

Relapse is possible in *P. vivax* malaria due to long-lasting dormant hypnozoites, which can remain dormant for months before reactivating [36]. If the current blood stage infection is cleared an individual may test negative with a routine diagnostic, therefore if hypnozoite carriers aren't treated appropriately a new transmissible blood stage infection can occur some time after the original infection. There are no diagnostic tests for hypnozoites, so it is near impossible to identify asymptomatic carriers. Asymptomatic *P. vivax* carriers are common as infections are typically of lower parasitaemia than *P. falciparum* infections [37]. Although these infections are low density, they have a high transmission potential due to gametocytes appearing early during the course of infection [38]. Studies have shown that gametocytes are present in the peripheral blood so early that mosquito transmission can occur even before the onset of symptoms [39]. Treatment of *P. vivax* infections encompass drugs that target both the blood stage and the liver stage. Primaquine is the only licensed drug to treat liver stage parasites

but its use is limited in areas of high G6PD deficiency. The G6PD enzyme protects red blood cells from oxidative stress, but some individuals are genetically deficient in this enzyme [40] and so treatment with primaquine (which places oxidative stress on RBCs and causes haemolysis) is not advised until the G6PD status of a patient is determined. Currently available G6PD tests have many limitations so treatment with primaquine is often avoided or given at low doses over extended periods. Changes in dosing decreases the likelihood of complete clearance of the liver stage infection, thus enabling subsequent relapse in patients where clearing was not achieved due to a suboptimal dose of primaquine.

Given that treatment of *P. vivax* infections can be difficult (especially if the G6PD status of an individual is not known), there are no diagnostic tests that can detect dormant hypnozoites, and transmission can occur readily before the onset of clinical disease, *P. vivax* infections remain a significant challenge for elimination efforts.

1.3.3 Transmission risk factors

As previously mentioned, individuals most at risk of malaria are those residing or working in forested areas. Such areas are usually hotspots for mosquitos and as such biting is much more frequent. Many people that live in these areas are also ethnic, marginalised, or otherwise vulnerable minorities and behavioural factors may further increase their risk of malaria, their contribution to the asymptomatic reservoir, or both. This includes factors such as lack of education, lack of adherence to intervention strategies, preference of traditional housing, and transient mobility patterns [24, 41]. As a result, these people are less likely to seek out, or even come into contact with, health care providers; thus can harbour parasites that are transmissible to mosquitos without intervention.

1.3.4 Gametocyte carriage and transmission

As mentioned in **Section 1.1.2**, gametocytes are the sexual stage parasites that are ingested by mosquitos when they take a blood meal, infect these mosquitos, and propagate the transmission of the disease. Gametocytaemia is important as it is often used as a surrogate marker for the transmission potential of certain populations, where directly measuring infectivity is not possible. Detecting gametocytes can be challenging in endemic settings as they cannot be detected by RDTs. They can be

detected by microscopy, but this can be difficult at low densities. Moreover, in species other than *P. falciparum*, gametocytes lack distinctive morphology and resemble other stages of the life cycle, so can be missed even at high densities [42]. Certain developmental stages of the gametocyte can also sequester away from the peripheral blood and can therefore be missed by diagnostics, depending on the time of testing. These characteristics make detecting gametocytes difficult which results in an inability to diagnose carriers of the transmissible form of the parasite. Detecting both circulating and gametocyte stage parasites may be beneficial as in some cases gametocytes are present when circulating parasites are not, therefore having a test that only detects blood stage parasites may miss some infections.

1.4 Current diagnostics for malaria

1.4.1 Clinical diagnosis

Malaria can be diagnosed using several technical approaches. Some methods are more commonly used than others, and choice of diagnostic is often dependent on the location or the patient's circumstances. When a patient first presents to a health care facility a clinician may make a clinical diagnosis. Such a diagnosis is based on a patient's symptoms, but early symptoms such as fever, chills, headaches, and nausea are not specific and are common in many other conditions [3]. In most instances, a malaria-specific diagnostic test is conducted prior to treatment, as misdiagnosis can result in serious morbidity or mortality.

1.4.2 Light microscopy

Light microscopy is the most commonly used diagnostic for malaria diagnosis in endemic countries. It involves a Giemsa stain of the patient's blood (which stains the parasite DNA purple) and examination under a microscope (**Figure 1.4 A**). Microscopy has the ability to determine the presence of malaria parasites, differentiate between species, and enumerate the parasitaemia (the percentage of the patient's RBCs that are infected by parasites) [3]. When performed by an expert, microscopy has a detection limit of approximately 50 parasites/µl [43]. However, there are many hurdles to achieving high quality malaria diagnosis using microscopy. Microscopes are expensive and can be easily damaged under field conditions. Remote and rural health care centres may not have access to microscopes, or may struggle with maintenance. Microscopy requires highly trained personnel to examine slides accurately. Inspecting slides is a time-consuming process that is affected by factors such as the thickness and the positivity of the smear prepared, the competency of the microscopist, and the condition of the microscope. Microscopists may have many other tasks to perform during the work day and should typically only dedicate a maximum of 4 hours a day to examining slides [44]. Any longer and fatigue may reduce the accuracy of the reads. To accurately examine a low-density positive or negative thick film, a minimum of 6 minutes is needed per slide [44]. It is therefore widely accepted by the WHO that a microscopist can read no more than 30-40 slides effectively per day [44]. Microscopy on a large scale is therefore not practical in pre-elimination settings as it would be too time consuming and laborious to screen large number of asymptomatic people with low-density parasitaemia.

1.4.3 Rapid diagnostic tests

RDTs are common tests used for malaria diagnosis and are suitable alternatives where reliable microscopy is not available. They are immunochromatographic tests that detect parasite antigens such as HRP2 and LDH. They are beneficial in resource limited settings as they are: easy to use, easy to interpret (i.e. determining infection on the basis of coloured bands being either present or absent- **Figure 1.4 B**), affordable, and results can be obtained quickly (15-20 minutes). While commonly used, they can be inaccurate, have a limit of detection (LOD) of only approximately 200 parasites/µl [43], and can be affected by environmental conditions such as temperature and humidity [3]. Recently, a new ultra-sensitive RDT (us-RDT) for *P. falciparum* has been developed by Alere/Abbott Diagnostics.

It is still based on the HRP2 antigen capture in a lateral flow format, but is reported to be 10 times more sensitive than conventional RDTs [45]. Studies have shown that while these new us-RDTs are more sensitive than conventional RDTs, they are likely to misdiagnose infections with HRP2 negative parasites [46].

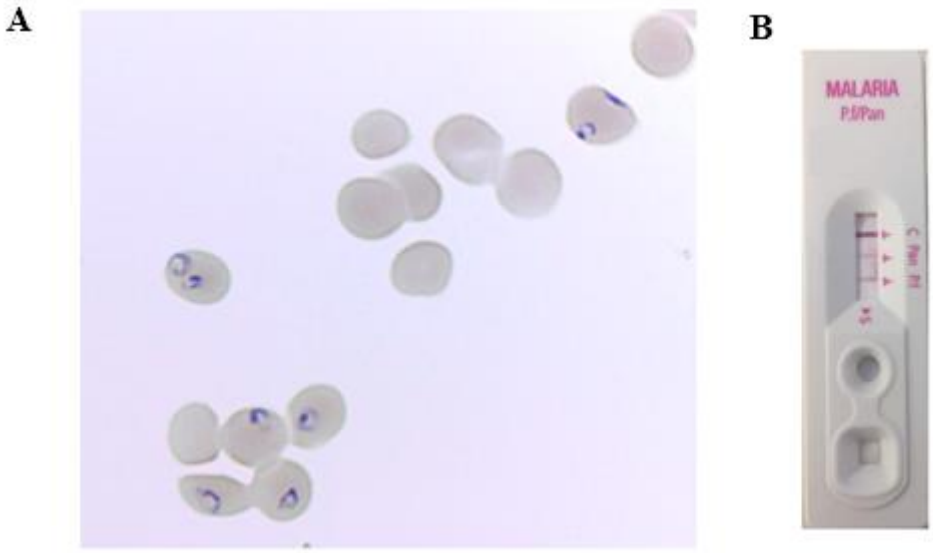

Figure 1.4: Detecting parasites with microscopy and RDT.
A high ring stage infection of 3D7 laboratory strain *Plasmodium falciparum* detected by microscopy and an RDT. **(A)** Giemsa-stained parasites can be seen inside infected RBCs, and **(B)** Pan (*Plasmodium* genus) and Pf (*P. falciparum* specific) test lines are apparent.

1.5 Molecular tests with malaria diagnostic applications

1.5.1 Potential of molecular based tests

Molecular diagnosis, while not as common as the aforementioned techniques, provide increased specificity and sensitivity. Nucleic acid amplification techniques (NAATs) are emerging as fast, sensitive methods for pathogen and resistance gene detection. NAATs have greatly enhanced infectious disease diagnosis in the past 15 years [47, 48]. Typically a sample contains the patient's own genetic material, as well as the genetic material of the infectious agent. NAATs work by amplifying, through replication, the nucleic acid sequence (most commonly DNA, but for some viruses RNA) of pathogenic microorganisms present in a patient's sample. Once amplified the nucleic acid can be detected,

commonly through direct visualisation in gels or spectrophotometrically [47]. Several methods of amplification exist, each having certain advantages and limitations.

1.5.2 Polymerase chain reaction

PCR is the most commonly used method of nucleic acid amplification. It has several applications, one of which is diagnostics. The PCR technique is extensively understood, and this enables reliable detection assays to be relatively low cost, and with adequate quality control.

PCR occurs in three distinct stages: 1) denaturation, where high temperature (94-96°C) is applied to separate the strands of DNA from one another and expose the primer binding sites; 2) annealing, where the forward and reverse primers bind to their respective sites; and 3) extension, where a new strand of DNA is formed from the addition of nucleotides by the combined action of the primers and DNA polymerase [49]. Essentially, target DNA from any organism can be amplified, provided that correlating primer sequences are used.

In addition to conventional PCR, real-time PCR (also referred to quantitative PCR, or qPCR) has become a widely used research and diagnostic tool in the past decade. The main difference between conventional and real-time PCR is that the latter can amplify the target DNA whilst simultaneously detecting the presence of the newly amplified DNA and can be used to quantify the amount of amplified product if appropriate standards are used [50].

Several studies have compared PCR to microscopy and RDT in malaria endemic settings and have found that a substantial number of infections are missed with these routine tests. A study in Tanzania saw a seven-fold increase in parasite prevalence in testing with qPCR compared to microscopy [51]. A study in Myanmar found positivity rates of 1.0% by RDT and 1.4% by microscopy, compared to 2.3% by PCR [52]. Along the China-Myanmar border, infections were detected in 1.2% of participants using microscopy, whereas conventional PCR detected 1.9%, and nested PCR detected 2.4% [53]. Even the improved us-RDT lack in sensitivity compared to PCR, missing 16% of PCR-detectable infections in a high-endemic malaria setting (Uganda) and 56% in a low endemic setting (Myanmar) [54].

Overall PCR (conventional and real-time) is a valuable diagnostic tool, and has definitive advantages over other techniques. But like any laboratory procedure, PCR is not without its limitations. Amplification can be adversely affected by various inhibitors, which dictates the need for DNA purification prior to amplification. DNA extraction and purification are time consuming and can add considerable cost to an assay and delay the time to result [55]. The major limitation of this technique in the context of its applicability as a diagnostic tool in low resource settings is that it requires expensive equipment, and is not portable, robust or fast enough to be considered a true point of care (POC) diagnostic [55]. As such, alternative techniques need to be developed to address these limitations and improve diagnostics as a result.

1.5.3 Isothermal amplification

Isothermal amplification methods are becoming increasingly common and useful in resource limited settings as they have many advantages over PCR. Assays such as thermophilic helicase dependent amplification, nucleic acid sequence based amplification, and recombinase polymerase amplification have all been assessed for malaria applications [56-58] but loop-mediated isothermal amplification (LAMP) is the most widely used isothermal amplification tool.

1.6 LAMP as a diagnostic tool for malaria

1.6.1 Principle of LAMP assay

LAMP is a relatively new technology, developed in 2000 [59]. It can be used to replicate DNA to levels where it can be easily detected, under isothermal conditions. LAMP assays are emerging as simple, low-cost and robust assays to detect pathogens without the need for laboratory infrastructure unlike other NAATs such as PCR.

To enable isothermal amplification, LAMP utilizes a strand displacing DNA polymerase, which separates double stranded DNA whilst simultaneously replicating it. RNA can also be amplified using LAMP, with the addition of a reverse transcriptase. Incubation temperatures range between 60 °C and 65 °C, for a duration of 15-60 minutes [60-63] four primers are usually used in LAMP reactions; the forward inner primer (FIP), backward inner primer (BIP), forward primer (F3), and backward primer

(B3). An additional two primers can be used, with the loop forward (LF) and loop backward (LB) primers used to accelerate the rate of reaction [63] LAMP primers are designed to specially recognise six regions of the target DNA, and amplification will not occur unless all six of these regions are identified.

The outcome of LAMP assays can be detected by post amplification gel electrophoresis [64-66] or direct DNA binding dyes. Contamination risks due to the cross-over contamination of LAMP amplicons in subsequent reactions are high as LAMP reactions generate up to 100 times more amplicons than PCR assays [61]. Alternatives to post-amplification processing typically rely on dyes binding to by-products of the amplification process, or by other changes caused by amplification such as changes in pH. By-products such as pyrophosphate are generated during amplification and bind to free magnesium ions in solution. This causes turbidity in the solution, and while this turbidity can be visualized by the naked eye the addition of a dye enhances visualization. Dyes such as calcein and hydroxynapthol blue can be used in this instance and will be discussed in later chapters.

Initial primer binding

LAMP can be thought of as occurring in cyclic and non-cyclic stages; a visual representation of this can be seen below in **Figures 1.5-1.7.** The first stage involves the primers selectively identifying and binding to each of the six target regions (**Figure 1.5)**. The FIP is the first primer to bind to its target site. It consists of an F2 region at the 3′ end, which is complementary to the F2c sequence on the template, and an F1c region at the 5′ end which is identical to the F1c region of the template. The F3 primer is the next to bind, it has a complementary F3c region to the target sequence. This primer is shorter and in lower concentration compared to FIP. BIP binds next and contains a B2 site at the 3′ end

(complementary to B2c) and B1c region at 5′ end (same as B1c on target). Finally B3 binds; it contains a B3 region complementary of the B3c region of the template sequence [2].

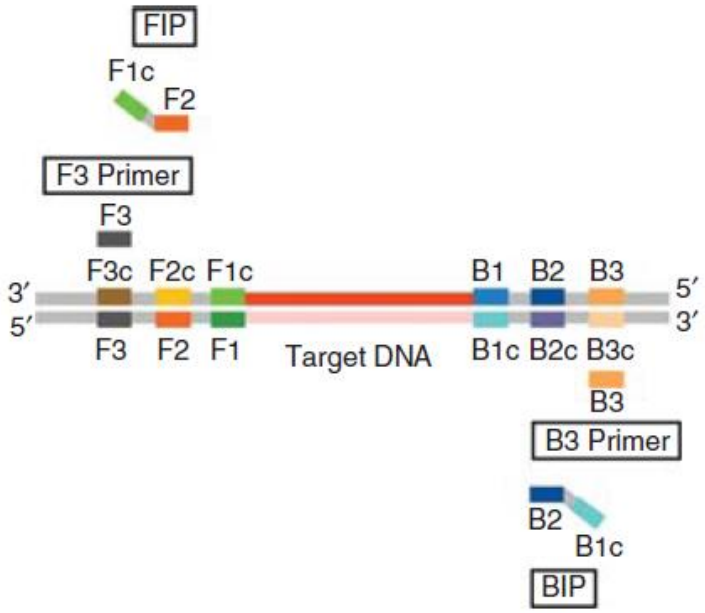

Figure 1.5: LAMP primers.
Each of the four LAMP primers can be seen this diagram. An example of a DNA target is also included to show the primers binding sites [2].

Amplification

The FIP primer hybridizes to the F2c region of the target and initiates the synthesis of a complementary strand. F3 hybridizes to F3c region and extends the target while displacing the FIP-linked complementary strand (Steps 1-3 of **Figure 1.6**). The displaced strand forms a loop at the 5' end due to the complementary bonding of the F1 and F1c regions (Step 4 of **Figure 1.6**). This strand now serves as a template for the BIP and B3 primers. BIP hybridizes the B2c region; the synthesis of DNA is initiated, a complementary strand is formed, and the loop at the 5' end opens (Step 4 of **Figure 1.6**). B3 then hybridizes to the B3c region, extends, and displaces the BIP linked complementary strand (Steps 4 and 5 of **Figure 1.6**). This results in the formation of a dumbbell shape of DNA due to complementary bonding (Step 5 of **Figure 1.6**). This structure then initiates the cycling part of LAMP.

FIP hybridises the loop of the structure, and the F1 strand is displaced, forming a new loop at the 3' (Step 5, 5a, and 6 of **Figure 1.7**). Nucleotides are now added to the 3' end of B1, displacing the FIP strand and again forming a dumbbell shape of DNA (Step 7 of **Figure 1.7**). The BIP primer hybridises in a similar pattern, displacing the loop and forming one at the other end (Steps 7, 7a, and 8 of **Figure 1.7**). Further synthesis occurs in each of these displaced strands in the same way (Steps 5a and 7a). This cycling occurs in steps 5-8 of **Figure 1.7**. As each product forms (Steps 9a and 10a of **Figure 1.7**), it joins to another product due to complementary sequences with the aid of the corresponding primer, binds together, and forms a long complex or chain of product (Steps 11 and 12 of **Figure 1.7**). This chain is what is detected at the end of the reaction. As the chain forms, a "waste" product also forms which is completely closed due to complimentary bonding, and so cannot replicate any further. This waste product is minimal in comparison to the total amount of amplicon and so rarely contributes to, or interferes with the detection of the amplicon [2].

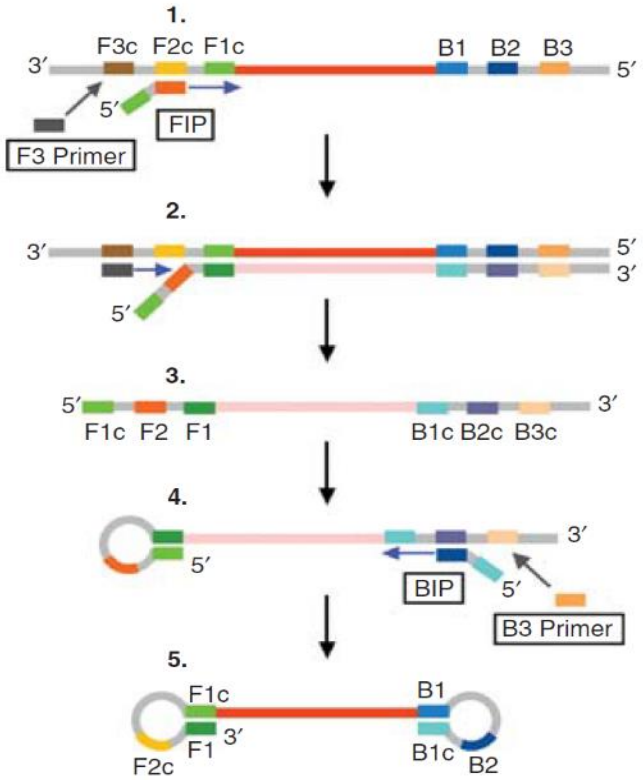

Figure 1.6: The non-cycling stage of LAMP.
The four primers bind to their specific target sites, displacing the strands as they go, and eventually forming the dumbbell structure that will be the starting structure of the cycling stage of LAMP [2].

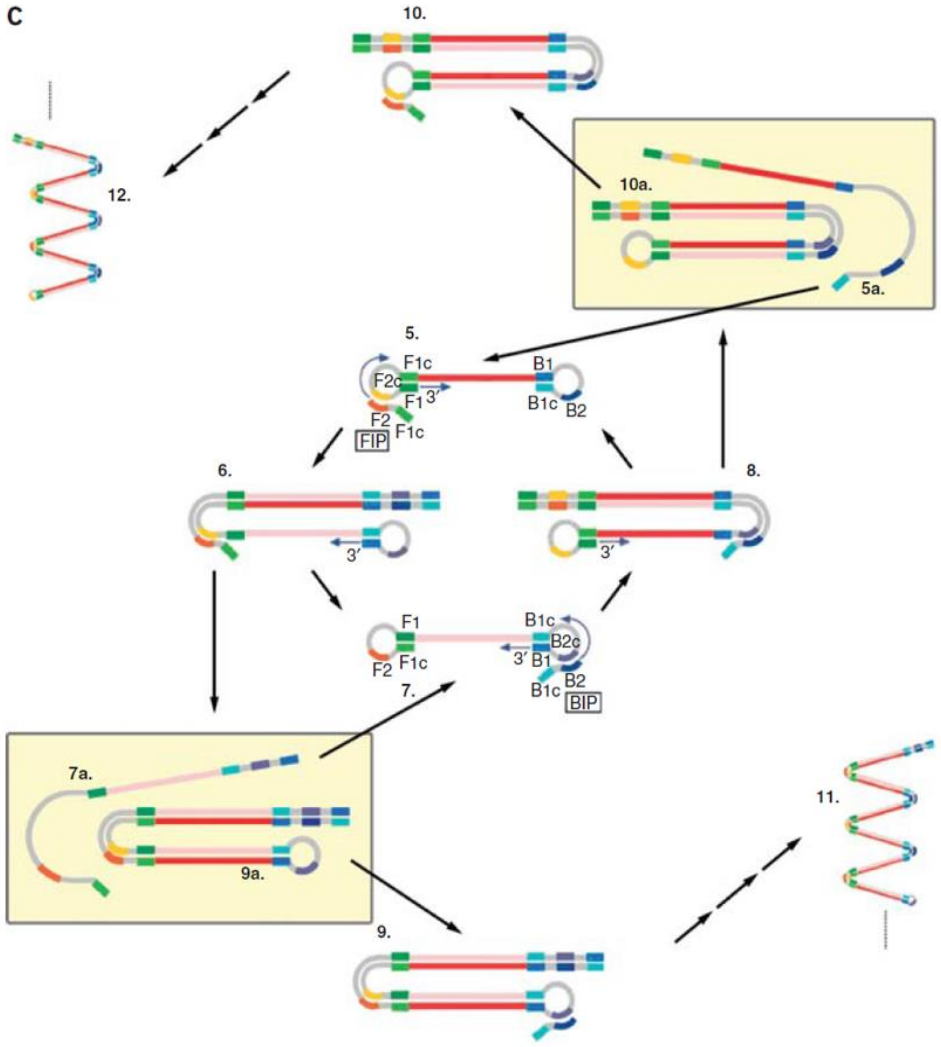

Figure 1.7: The cycling stage of LAMP.
The dumbbell structure formed in the non-cycling phase of LAMP is used at the starting structure in this stage. A long chain of product forms by amplicons binding to one another, and a waste product that cannot be amplified further is also produced [2].

1.6.2 LAMP compared to other NAATs

Although both PCR and LAMP are more sensitive diagnostic tools than microscopy and RDTs, LAMP is more feasible in resource limited settings. As amplification occurs isothermally, complex thermal cycling instruments are not required. Standard laboratory equipment such as heat blocks and water baths are sufficient and are much more durable and portable than PCR thermocyclers. PCR assays are very susceptible to biological inhibitors in clinical samples and thus DNA purification is critical prior to processing. However these techniques may be outside the expertise or infrastructure level of field settings. Simpler sample processing is adequate for LAMP testing as it is very tolerant to these same inhibitors.

1.6.3 LAMP assay compared to other malaria diagnostics

LAMP has distinct advantages over widely used malaria diagnostics. Once established, a LAMP assay would be much simpler to perform than a microscopy examination and could be carried out by a minimally-trained technician if necessary. For health workers with only elementary science knowledge, the WHO recommends a 4-5 week microscopy training course, whereas studies that have implemented LAMP found that 3 days of LAMP training was sufficient [67, 68]. The time taken to perform LAMP assays would be much shorter compared to microscopy especially in low transmission settings where parasitaemia is low, and LAMP is not subject to operator variation or bias like analysing numerous slides in short succession can be. Like microscopy, LAMP requires very little equipment. However LAMP equipment may not require servicing or calibration to the extent microscopes do [69]. When compared to RDTs, LAMP is far more sensitive and accurate. LAMP is substantially also less susceptible to temperature and humidity fluctuations and LAMP assay outcomes are can be less ambiguous than RDT results particularly in weak positives.

1.6.4 LAMP assay for malaria

To date, LAMP has been used to detect many diseases and has been found to be particularly useful in malaria endemic settings as it is simple, cost effective, and requires little resources. Generally LAMP assays have been designed to target the small subunit ribosomal RNA (18S rRNA) [70] or the mitochondrial DNA (mDNA) [71], but targets have also included apical membrane antigen 1[72] and ß-tubulin [73].

A commercial malaria LAMP assay has been developed by Eiken Chemical Company, Japan. These kits contain a lyophilized proprietary formulation of LAMP reagents in individual reaction tubes. A simple DNA extraction is performed on a biological sample and this extract is used to reconstitute the reagents. Reaction tubes are then incubated and results detected by fluorescence under UV light. This kit is very expensive at around US$14.50 per test. Several studies have tested these kits in the field with success [74-76] but the cost per test has limited their scale-up in low-middle income countries. Solely relying on visual detection can also be a challenge, especially when performed by inexperienced staff.

As a result, researchers have sought to develop their own LAMP assay to mitigate cost and improve detection accuracy. Lu and colleagues (2011) compared their "in-house" *P. vivax* LAMP assay to the Eiken LAMP kits and found no difference when tested on clinical samples in central China. Singh and colleagues developed *Plasmodium* genus and species-specific LAMP assays that allowed for distinct visual discrimination between positive and negative samples and compared the test to PCR and microscopy. Patel and colleagues (2014) developed a LAMP assay with real time monitoring capabilities which cost approximately US$2.66 per test. They trialled this assay in India and Thailand and found it was comparable to PCR with a sensitivity of 94.8% and 100% specificity. While all these assays are much cheaper than the commercially available alternatives and compared well to the PCR gold standard, none addressed the major hurdles that prevent in-house LAMP assays from truly becoming POC tests, such as multi-step work flows and dependence on cold chain storage.

1.7 Aims of this thesis

This thesis focuses on improving the sensitivity of malaria diagnostics in order to achieve malaria elimination and is based on three main premises: (1) there is a global shift from controlling malaria to eliminating malaria; (2) current malaria diagnostics like microscopy and RDTs are not sufficiently sensitive to detect asymptomatic carriers who are an important reservoir for transmission; and (3) currently used high sensitivity molecular tests that are able to detect asymptomatic carriers are not suitable for use in low resource settings and so are not appropriate in an elimination context.

The central hypothesis of this work is that LAMP is a practical tool to address the unmet need of a more highly sensitivity diagnostic test for detection of asymptomatic infections in elimination settings.

The aims of this thesis are:

1. Assess the challenges associated with implementing LAMP technology in a remote and resource limited setting as part of a National Malaria Control Program activity (Chapter 3)
2. Assess the practicality of LAMP as a field-based test by implementing the commercial LAMP kit in a resource-limited field setting (Chapter 4)
3. Develop and validate an in-house LAMP assay that is comparable in sensitivity to commercially available LAMP kits and gold standard qPCR assays (Chapter 5)
4. Further develop the LAMP technology to meet practical diagnostic requirements for wide-spread applications (Chapter 6)

Malaria elimination is an achievable goal in many parts of the world, but requires improved diagnostics. This work focuses on improving malaria diagnostics by increasing sensitivity and field applicability. Ideally, all parasite infected individuals could be detected and treated, thus preventing further transmission and working towards malaria elimination.

2 Materials and Methods

2.1 The Regional Artemisinin-resistance Initiative (RAI) study in Vietnam

The RAI study was conducted in South East Vietnam in Binh Phuoc and Dak Nong Provinces. The annual entomological inoculation rate in these areas of *P. falciparum* was 2.2 infective bites per person-year. The main *P.falciparum* vectors are *An. dirus s.s*. and *An. minimus s.s*. The *P. vivax* annual entomological inoculation rate was 2.5 infective bites per person-year. The main *P. vivax* vectors are *An. sawadwongporni*, *An. dirus s.s.* and *An. pampanai* [17].

The first phase of the study involved household mapping in three communes (Dak Nhau, Bu Gia Map, and Dak O) of Binh Phuoc Provinceand in two communes (Dak Ngo and Quang Truc) of Dak Nong Province (**Figure 2.1**). During a 3 week period approximately 11,000 households were geographically mapped using mobile phones and data was collected on malaria interventions currently in place (i.e. IRS, ITN, both or none). The second phase of the study was a cross-sectional survey that sampled and collected data from individuals in those same communes. All data was collected using paper forms that were later uploaded to online data collection software Epicollect 5. Demographic information (age, gender), house location (village level), and the results of RDT (if symptomatic at time of testing), microscopy, and LAMP testing were recorded on these forms. A total of 5,421 participants were sampled over a 3 month period for the cross-sectional survey. **Table 2.1** below shows a breakdown of the samples collected during this study.

Figure 2.1: Study sites selected for RAI cross-sectional survey
Two provinces in South East Vietnam were selected; Binh Phuoc and Dak Nong and samples were collected in five communes across these two provinces.

Table 2.1: Demographic details of study participants.

Total sampled		
	Bu Gia Map	n=1415
	Dak Nhau	n=1100
	Dak O	n=1364
	Dak Ngo	n=1367
	Quang Truc	n=175
TOTAL		**n=5421**
Age (overall)*	median (range)	24 years (1-97 years)
Gender (overall)*	female	n=3132 (54.4%)

* Some participants had incomplete data sets and thus were excluded from this analysis (n=377)

2.1.1 Sample collection in villages

Sampling in each commune was carried out throughout the duration of study. Collection points were established at a central locations in selected villages; these locations included the households of prominent village figures and outside schools and churches, and sampling was conducted on different days of the week and times of day to ensure an accurate representation of the population. Samples were collected from a finger prick; the first drop of blood was wiped away. The second drop of blood was used to make a thick smear for microscopy. A sample of at least 200 µl was collected in heparin-containing microtainers (Becton Dickenson) where possible (i.e. less than 200 µl was collected from some individuals). If symptomatic at the time of testing, a Malaria Ag P.f/P.v RDT (SD Bioline) was also performed and if positive the participant was referred to the health care centre. Pre-printed barcodes were used to identify different samples from the same participant. After collection, samples were transported to the nearest health care centre for further testing.

2.1.2 Sample processing at Commune Health Care Centres

Microscopy

Thick smears prepared at collection sites were fixed in absolute methanol and stained with 10% Giemsa solution for 5 minutes, prior to being examined by an expert microscopist. Smears were reported as positive or negative and species was identified where possible, but parasite density was not recorded.

Dried blood spot preparation

Three 20 µl spots of blood from the microtainers were spotted onto pre-cut pieces of 3MM filter paper (Whatman) (7 mm x 3 mm). Dried blood spots (DBS) were left to air dry for a few hours, before being packaged in zip lock bags with desiccant and frozen. At the conclusion of the study, DBS were transported to Australia for further testing.

LAMP testing

DNA was extracted from all samples collected prior to testing with LoopAMP MALARIA Pan kits (Eiken Chemical Company, Japan). This involved the "boil and spin" method, in which 60 µl of sample added to 60 µl of extraction buffer (400 mM NaCl, 40 mM Tris pH 6.5, 0.4% SDS) and incubated at 95 °C. After 5 minutes, samples were centrifuged at 10,000g for 3 minutes, then 30 µl of supernatant was diluted in 345 µl nuclease free water (NFW). A 30 µl aliquot of this dilution was added to a LoopAMP tube as DNA template. Tubes were inverted and incubated for 2 minutes (to reconstitute the dried reagents in the lid of the tubes), and were then mixed to ensure sufficient reconstitution. Tubes were then incubated in a 65 °C heat block for 40 minutes. The reaction was terminated by incubating at 80 °C for 5 minutes. Tubes were then observed under UV light for fluorescence and results recorded.

2.1.3 Sample processing in Australia

All samples were transported to Australia and subset of the DBS samples (n=500) were used for DNA extraction and tested with PCR. This subset included all LAMP positive cases (n=70) and a 1:3 proportion of negatives (n=430) from the same area. For example, if 10 positive cases were found in Bu Gia Map, then 30 samples that were reported negative from that commune, were selected at random and tested by PCR.

DBS extraction

DNA was extracted using the Tissue Genomic DNA Extraction Mini Kit (Favorgen) as per the manufacturer's instructions. In brief, two 3mm punches were incubated at 85 °C for 10 minutes in 200 µl FATG1 buffer. Then, 20 µl proteinase K was added and samples were incubated at 60 °C for 3 hours vortexing every 40 minutes. Samples were incubated in 200 µl FATG2 buffer for 10 minutes at 70 °C, and 200 µl of ethanol was added before the liquid mixture (excluding punches) was added to spin columns. W1 buffer (400 µl) was washed through the column by spinning at 18,000g for 1 minute, followed by another wash with 750 µl of wash buffer, and a final spin to remove all residual buffers before eluting in 100 µl elution buffer.

qPCR testing

Extracted DNA from the 500 DBS samples were tested by real time PCR (QuantStudio 7) as a gold standard comparison to the LAMP testing carried out in the study. Samples were screened with a *Plasmodium* genus assay, with any positives subsequently tested in *P. falciparum* and *P. vivax* specific assays. These assays target the 18S ribosomal RNA of the parasites (**Appendix 1**). Each reaction was made up of 10 µl 2x SensiFAST Probe No-ROX One-Step mix (Bioline), 0.6 µl reverse primer (RP), forward primer (FP), and probe, 3.2 µl NFW, and 5 µl sample. Cycling parameters for the assay were 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 15 seconds and 60 °C for 1 minute. Fluorescence was read in the FAM channel. This protocol was kindly provided by Ms Franca Azzato (Victorian Infectious Diseases Reference Laboratory). This protocol is not widely published but is a standard protocol used by VIDRL for testing all samples from returned travellers.

2.2 General protocol for parasite culture

2.2.1 Growing and monitoring

The D10 and 3D7 strains of *P. falciparum* were cultured according to standard techniques [78]. Media comprised of RPMI-HEPES with 0.5% Albumax and 0.2% sodium bicarbonate (Gibco), and O^+ blood at a ~4% haematocrit, was used to culture parasites in a 10 ml culture dish. Dishes were placed in perspex boxes and filled with a gas mixture of nitrogen (95%), carbon dioxide (4%), and oxygen (1%) and incubated at 37 °C either stationary or on a shaker to promote single invasions. Parasite density was measured by examining thin smear slides using light microscopy. Smears were made by adding and then spreading 2 µl of blood onto a glass slide (Menzel-Glaser), fixing the slide with absolute methanol, and then staining with 10% Giemsa solution (Merck) for 10 minutes. The parasitaemia was then calculated by counting the percentage of parasite infected red blood cells.

2.2.2 Synchronising to ring stage before harvesting

Parasites were synchronized at ring stage using sorbitol (Sigma). The ring stage of the parasite was used for most experiments because this is the stage that is seen in circulation during *P. falciparum* infections (i.e. other blood stages sequester in peripheral circulation). This is important because ring stage parasites have less DNA than later trophozoite or schizont stages. To synchronise parasites, 10 ml of media and parasite culture were resuspended and then centrifuged at 1500 rpm for 5 minutes at 20 °C. Spent media was then removed and replaced with 10 ml of pre-warmed 5% sorbitol, then incubated in a 37 °C water bath for 5 minutes. Cells were then centrifuged again at 1500 rpm for 5 minutes at 20 °C and the sorbitol removed before resuspending parasites in new media.

2.3 General protocols for DNA extraction

2.3.1 Parasite harvest and parasite titrations

Once cultures reached the desired parasite density, they were again treated with sorbitol to ensure the presence of ring-stage parasites only. This parasite density was used to calculate a 1% dilution using a mixture of parasitized blood from culture and non-parasitized blood in the form of packed red blood cells from healthy donors. This dilution was then further titrated out to 0.00001% in ten-fold increments. The dilutions were as follows; 1%, 0.1%, 0.01%, 0.001%, 0.0001%, and 0.00001%, which represented 50,000 parasites/µl, 5000 parasites/µl, 500 parasites/µl, 50 parasites/µl, 5 parasites/µl, and 0.5 parasites/µl, respectively. These dilutions were used to generate a standard curve and determine the LOD and sensitivity of the assay.

2.3.2 Whole blood extraction

Boil and spin method

As previously described in **Section 2.1.2**

Kit extraction

DNA was extracted using the Tissue Genomic DNA Extraction Mini Kit (Favorgen) as per the manufacturer's instructions. In brief, 200 µl of sample (50 µl whole blood+150 µl of 1X PBS) was added to 20 µl proteinase K and incubated at 60 °C for 30 minutes (mixing every 10 minutes). Samples were then incubated in 200 µl FATG2 buffer for 10 minutes at 70 °C, and 200 µl of ethanol was added before the liquid mixture was added to spin columns. W1 buffer (400 µl) was washed through the column by spinning at 18,000g for one minute, followed by another wash with 750 µl of wash buffer, and a final spin to remove all residual buffers before eluting in 100 µl elution buffer. This method is similar to that described in **Section 2.1.3** (DNA extraction from DBS) but was used on whole blood samples only.

2.3.3 DNA quantification

All extracts were quantified before use by analysing in the Quantus fluorometer (Promega) following the manufacturer's instructions. In brief, the Quantus was calibrated by reading a blank sample (200 µl of QuantiFluor ONE dsDNA dye) followed by a 400 ng standard sample (2 µl of QuantiFluor ONE Lambda DNA standard to 400 µl of QuantiFluor ONE dsDNA dye). DNA extracted samples were then prepared by adding 2 µl to 200 µl of QuantiFluor ONE dsDNA dye and were incubated at room temperature for 5 minutes before reading.

2.4 General protocols for LAMP assay

2.4.1 LAMP reaction mix

Each LAMP reaction was comprised of isothermal buffer, $MgSO_4$, dNTP mix, *Bst* WarmStart 2.0 polymerase, NFW, and *Plasmodium* genus or *Plasmodium falciparum* specific primers. Concentrations of $MgSO_4$, dNTP mix, and individual primers in primer pools were optimized for sensitivity and specificity (detailed in **Chapter 5**). The optimized LAMP reaction mix contained the following; 2.5 µl 10× isothermal buffer (NEB), 1.5 µl 100 mM $MgSO_4$ (NEB), 3.5 µl 10 mM dNTP mix (NEB), 1 µl 8000 U/µl *Bst* WarmStart 2.0 polymerase (NEB), and 7 µl NFW. A primer pool was made up of 1.6 µM FIP and BIP, 0.2 µM F3 and B3, and 0.8 µM Loop F and Loop B (Geneworks), and 5 µl of this was also added to each reaction. Primers targeted either the 18S rRNA or mDNA regions of the *P. falciparum* genome, the sequences of which can be found in **Appendix 1.** In addition, the duration and temperature of the LAMP reaction was also optimized.

2.4.2 LAMP running conditions

Experiments were either carried out in a T16- ISO instrument (Axxin) or in a conventional PCR instrument (for higher throughput). If conducted in the T16, running conditions were 65 °C for 60 minutes and tubes were removed immediately after the incubation period had ended. The T16 is not equipped for a high temperature denaturation step, thus if tubes were not physically removed they would continue to incubate and non-specific amplification may result. If conducted in the PCR instrument (QuantStudio) thermal cycler, running conditions were 65 °C for 60 minutes with an 85 °C termination step for 5 minutes. The same running conditions (65 °C for 60 minutes) were performed when using low technology incubators such as the heat block.

2.4.3 LAMP amplicon detection

SYBR Green

As previously mentioned, SYBR Green inhibits *Bst* and therefore amplification. SYBR Green should therefore only be added after amplification for this application. A dilution of 1:4 was made up with dH_20 from a stock solution of 10,000x concentrate in DMSO (Sigma-Aldrich). This working stock was made fresh before each experiment and 1 µl of it was added into each reaction at the end of the amplification. A visual colour change was observed with positive samples turning green and negative samples remaining orange.

Calcein

Calcein was combined with $MnCl_2$ to remain in a quenched state. Optimal concentrations of each reagent were determined by a serial dilution. Stock concentrations of 500 µM, 250 µM, and 125 µM of $MnCl_2$ (Sigma Aldrich) and 250 µM, 125 µM, and 62.5 µM of calcein (Sigma Aldrich) were prepared. The final concentration of each in a 25 µl reaction equated to 10 µM, 5 µM, and 2.5 µM of $MnCl_2$ and 5 µM, 2.5 µM, and 1.25 µM of calcein respectively. Optimal concentrations of both reagents were prepared in advance by combining each in equal parts. This mixture was stable at room temperature, so was made and used for several subsequent LAMP reactions, stored away from light in between experiments. A 2.5 µl portion was added to each LAMP reaction prior to amplification. After addition, samples were orange in colour. Positive samples turned green post-amplification, with negative samples remaining orange.

T16 fluorescent detection

When not performed in a conventional PCR thermocycler, LAMP assays were run in the T16- ISO (Axxin). The T16, is an isothermal instrument that is compact, portable, and can operate in stand-alone mode. It can house up to 16 LAMP tubes per run and has three channels of fluorescent detection. Desktop software can be used to create assay parameters, configure settings, detect amplification in real time, and can support 2 instruments at once (allowing for 32 tests per run). The stand-alone function allows operation without being connected to a computer and displays results (positive or negative) on an LCD screen at the end of the run. The three fluorescent channels equipped on the instrument are FAM, HEX, and ROX and the channel used depends on the dye incorporated into the LAMP reaction. The formulation of the LAMP assays was designed for a visual read of amplified product under a UV light. This meant that the levels of fluorescence were too high for the standard optics configuration of the T16 instrument (i.e. FAM channel was saturated). After discussion with Axxin, a modification was made to the instrument to decrease the reading aperture in the FAM channel to enable reading of the calcein dye in the appropriate channel. Subsequent experiments were all recorded in this modified-FAM channel. The emission intensity of the LED was also altered (optimal at 18% intensity).

2.5 Statistical analysis

For mapping activities, StataSE (StataCorp LLC) was used to clean original data sets, and QGIS (open source) was used to create maps and overlay findings.

For evaluating sensitivity and specificity of LAMP compared to PCR and microscopy, the Wilson Brown method was used and Fishers exact test was used to determine significance.

For quantifying PCR results, a standard curve (fluorescence of each standard against Ct value) was transformed and analysed with a linear regression model. The transformed values of the samples were interpolated from this curve (omitting points deemed to be outside of the LOD) and then back transformed to obtain the parasite density of samples (**Appendix 5**).

For assessing the difference between dispensed volumes in T1 cartridge, unpaired, parametric t-tests or Holm-Sidak methods were used. For Holm-Sidak method, each row was analysed individually, without assuming a consistent standard deviation.

For melt curve analysis, curves were normalised by averaging sub-columns and normalizing means, defining 0% as the smallest value in each data set and defining 100% as the largest value in each data set. The first derivative was found from normalised curves, smoothing to four neighbours on each side of the average. The left Y axis of the figure was then reversed, showing negative values.

2.6 Software used

Epicollect 5 (Imperial College London) was used for collection of field data, Microsoft Excel (2010 Microsoft Corporation) was used for data collection and organisation, and Prism 7 for Windows (GraphPad Software, Inc.) was used for statistical analysis and to make figures.

2.7 Ethics and informed consent processes

Ethical approval for this study was obtained from the Ministry of Health of Vietnam and from the ethics committee of the National Institute of Malariology, Parasitology, and Entomology. All study participants provided written informed consent.

3 Implementation of LAMP for mass screening in malaria elimination settings

3.1 Introduction

Malaria control efforts traditionally focus on diagnosing and treating symptomatic cases of malaria. It is known however, that even asymptomatically infected people can be a source of parasites for mosquito transmission. In order to fast-track malaria elimination, increased emphasis has been placed on finding and treating these asymptomatically infected individuals [22, 23, 79]. There are several additional challenges to be overcome when seeking to diagnose this group compared to those with symptomatic malaria. Firstly, these are well individuals that are living in their community and are not usually presenting to health services, so diagnostic strategies need to consider workflows for mass population engagement, sampling and testing. Secondly, asymptomatically infected individuals often have lower parasite densities, which are below the LOD by standard diagnostic approaches like RDTs and microscopy. Thirdly, within a malaria elimination setting, it might be expected that a small number of people will be infected. Therefore, it is likely that there will be a large number of people that will need to be tested, but with a small number of positives expected. This has important implications for the sensitivity, specificity and cost of the test being deployed. LAMP is a technology that has the potential to detect asymptomatic individuals with low parasite densities at low cost. This methodology has been described in detail in **Chapter 1**, but is essentially an isothermal nucleic acid amplification strategy. Although this technology holds potential for such an application, there are a lack of studies assessing the feasibility of implementing LAMP as part of National Malaria Control Program (NMCP) activities.

The successful introduction of any new diagnostic tool depends on a range of factors that are external to the performance of the test itself. Some of these factors relate to issues of health policy, funding and the health system itself, including the infrastructure required to scale up the use of a new diagnostic test [80]. This includes the capacity to procure and distribute tests and associated equipment. The willingness to undertake locally-relevant research and development of diagnostic tools is also critical. Policymakers sometimes fail to prioritise diagnostic test research, thus failing to gather the body of

evidence required to show efficacy [81]. It is also the case that diagnostic test development usually doesn't receive the same amount of attention and funding as drug development [82].

Another key determinant of successful adoption of new diagnostics depends on factors relating to the health staff that implement these new diagnostic tools [81, 82]. Such factors include test-specific issues like: staff understanding the principles of the test and the key differences with other diagnostic approaches; acceptability of the test approach; the adequacy of training and ongoing support to the staff; and the logistical requirements to engage with the test recipients, especially if this requires mass sampling [81]. There are also factors relating to how the test is done by the staff, including the work flow of the test and impact that this has on other tasks and responsibilities; technical competency of the staff in sampling, conducting the test, and interpreting results. The processes required for data entry are also crucial and is often a balanced interplay between simple manual systems like paper-based data recording and newer technologies that enable digital data entry which remain unfamiliar to some end-users. These factors do not affect test efficacy or performance of the test itself, but can have a dramatic impact on the effectiveness [83].

The successful implementation of a new tool in a field setting is as important as the performance of the tool itself. In order to truly make an impact, a diagnostic tool must be adopted and successfully integrated into that specific health care system. Studies have investigated the importance of developing tools that explicitly consider end user requirements [80] and ensuring capacity building of community health care workers [82]. Implementers may be unable to overcome context specific barriers without considering the perspectives of field staff as they play a key role in the successful adoption of new technology [84, 85].

Some of the issues outlined above are explored in this chapter in the context of a pilot study that sought to implement LAMP in a remote region on the Vietnam-Cambodia border. These activities were conducted as part of malaria elimination activities carried out by the NMCP of Vietnam. As part of this PhD, I helped to develop the framework for the rollout of LAMP with the NMCP, assisted in the training and field implementation, and conducted a preliminary assessment of this rollout across five communes. In addition to exploring some of the factors that can influence successful uptake of new

technologies, like LAMP, this chapter also seeks to set the broader context for LAMP data presented (**Chapter 4**) and some of the requirements that need to be considered in the design of an in-house LAMP assay (**Chapter 5**).

3.2 Methods

The study sites and protocols for this study have been previously outlined in **Section 2.1**, with further information provided below.

3.2.1 Commune Health Care Centres

Each of the five Commune Health Care Centres participating in the study typically had one Centre Head and two or three Health Care Workers. Each village typically had its own Village Health Care Worker.

The infrastructure of each Commune Health Care Centre differed depending on location and the funding available; some Commune Health Care Centres were donated by international research institutes and were therefore better equipped than Commune Health Care Centres in more rural areas (**Figure 3.1**). However, all Commune Health Care Centres had reliable electricity, beds for overnight patient stays, and diagnostic equipment such as microscopes. Some Commune Health Care Centres were equipped to treat patients with an in-house pharmacy. All Commune Health Care Centres were centralised within the populations they serve and easily accessible to members of the community. None of the Commune Health Care Centres were equipped for sample processing and LAMP testing, so all equipment was shipped from Australia or purchased in Vietnam prior to the study.

Figure 3.1: Commune Health Care Centres in Binh Phuoc Province
Commune Health Care Centres in Dak Nhau (top) and Dak O (bottom)

3.2.2 Development of Standard Operating Procedures

A SOP for the implementation of the Eiken LAMP assay had already been developed by collaborators at FIND. This SOP was revised and refined for application in the field setting of this study, and was tested with NIMPE staff prior to use in the study. A list of the SOPs and study documents developed are listed below and can be found in **Appendix 2**:

- **SOP1: LAMP preparation.** This SOP was used when preparing extraction buffers and assembling sample collection kits
- **SOP2: Site setup.** This SOP was used to set up LAMP testing areas at Commune Health Care Centres
- **SOP3: Sample collection.** This SOP was used when collecting samples in villages
- **SOP4: LAMP testing.** This SOP was used for DNA extraction and LAMP testing of collected samples
- **SOP5: RDTs, microscopy, and DBS preparation.** This SOP was used if an RDT was necessary, when preparing samples for microscopy analysis, and when making DBS for further testing
- **SOP6: Crossectional form.** This SOP was used when filling out participant forms and uploading data to Epicollect
- **SOP7: Contamination management**. This SOP was used to prevent contamination from occurring and how to manage it if it did
- **LAMP reaction record sheet.** This form was filled out during each LAMP run and linked to Epicollect entries
- **Summary of workflow schematic.** This schematic was an overview of the study workflow and was used as a reference tool at the Commune Health Care Centres.
- **Cross-sectional form.** This paper form was used to collect participant data at sample collection sites and was later uploaded to Epicollect.

3.2.3 Training of NIMPE staff and subsequent evaluation

The National Institute of Malaria, Parasitology and Entomology (NIMPE) oversees all malaria control activities in Vietnam. NIMPE staff training on the use of LAMP was carried out over two days (30th-31st August 2017) at the NIMPE headquarters in Hanoi, Vietnam. Key observations regarding the strengths and challenges of this training program were documented and used to improve subsequent training sessions.

The principals of the project and the technical details of sampling and LAMP testing were presented by English speaking demonstrators and then translated to Vietnamese throughout the training session. English proficiency varied widely among the fifteen staff from NIMPE's molecular and field epidemiology departments. SOPs were read through and each step demonstrated by Burnet staff (henceforth referred to as "trainers"). NIMPE staff then practiced the same techniques in small groups with mock samples and LAMP reagents.

After all staff members had performed the complete workflow with multiple samples, feedback on the training, SOPs, and general workflow was obtained from training participants through a focused group discussion.

At the completion of the training, feedback was sought from the trainers on all aspects of the training. The feedback sought to capture the observations of the trainers, focusing on strengths and weaknesses of the training program.

3.2.4 Training of rural health care staff

Training for rural health care staff took place in a conference room of a hotel in Binh Phuoc Province over two days (6th-7th September 2017). District and commune level Health Care Workers from both Binh Phuoc and Dak Nong Provinces were in attendance. The technical proficiency of these staff members before the training was unknown and presumed to be minimal, so laboratory basics (i.e. pipetting and aseptic technique) were demonstrated alongside LAMP workflow. A training video was shown demonstrating the workflow, followed by the demonstration of each step of the revised SOPs. Directives from English speaking demonstrators were translated to Vietnamese throughout the training

session as English literacy was minimal. Key observations regarding the strengths and shortcomings of this training were documented.

At the completion of the training, trainers conducted a review of the training, as outlined in **Section 3.2.3**.

3.2.5 Data entry

The online data management tool Epicollect 5 was used in this study. An appraisal of the applicability of Epicollect 5 to this study was conducted. These approaches are outlined further in the results below.

3.2.6 Logistical considerations

At the completion of the study key study personnel, including the study PI and the PhD candidate assessed the key logistical constraints encountered in the study.

3.2.7 Feedback surveys from LAMP operators

In order to gauge the feasibility of LAMP in field settings and the acceptance of LAMP by end users, face-to-face, in-depth interviews were conducted with three primary LAMP operators during the study (they had completed their training and had processed numerous samples at the time of the survey). All operators were NIMPE staff, two had field epidemiology backgrounds and had never performed laboratory tests like LAMP before; and the other was an operator familiar with laboratory tests and had prior experience with LAMP. The questions asked pertained to the use of LAMP, understanding of LAMP, and effectiveness of training. The questions were written in English and delivered orally in Vietnamese by a translator. The operators' responses were digitally recorded and later transcribed into English. Due to the small sample size and informal structure of the surveys, data analysis beyond identifying major themes, was not possible. In order to thoroughly assess implementation, future studies would gather information from all trained operators (therefore obtaining feedback from operators with all levels of experience) and survey questions would beconsist of closed-ended matrix type questions to increase analytics.

3.3 Results

3.3.1 Evaluation of NIMPE staff training

The first phase of LAMP implementation as part of the NMCP of Vietnam required training of NIMPE staff. This included fifteen staff from NIMPE's molecular and field epidemiology departments. The regular roles of these staff members included data and statistical analysis, microscopy, PCR assay design and evaluation, ELISA assay design and evaluation, antimalarial resistance assay development, clinical practice, and administrative and logistical roles. Only two of the fifteen staff members were familiar with LAMP and had used it before, and approximately half of the staff members had general laboratory experience. An assessment conducted by the trainers at the completion of the training identified some key potential impediments to the training and to effective uptake of LAMP within the NMCP (**Table 3.1)**. The two main issues that were identified were the relevance of the training for some participants, and the need to maximise the efficient use of time during the training.

Table 3.1: Potential impediments to effective uptake of LAMP as identified during NIMPE staff training

Impediment	Implications for staff training and field implementation
Staff with varying degrees of laboratory proficiency attended the same training session	Difficult to set the pace of training: too slow and experienced staff lose in interest and become distracted, too fast and less experienced staff get confused
Training of staff who are unlikely to directly perform or supervise LAMP	Difficult to focus training solely on key staff that would be participating in the implementation and ensure their proficiency
Extended discussions decreased time available for practical training	Staff did not have sufficient time to practise the workflow and trainers did not have sufficient time to address concerns or obtain sufficient feedback from staff
Non-essential but beneficial training aspects were omitted due to time constraints	Staff were not trained on how to correctly fill out participant forms and were not able to practise the workflow on multiple samples simultaneously

3.3.2 Evaluation of rural health care staff training

A second stage of training was done for field implementation staff. These Health Care Workers were from provincial, district, and commune health care levels. These Health Care Workers were well versed in RDTs and microscopy, but had little knowledge of other laboratory procedures. The training was structured to build on the existing knowledge of the Health Care Workers, focusing on the basics of laboratory work such as using pipettes to ensure that all staff members mastered the basics before moving onto the more complex LAMP procedures. Discussing the rationale for the SOPs was avoided during this stage of the training in the interest of time and this was found to be very effective as staff found it easier to follow a set protocol. This training session was more structured and consisted of a trainer demonstrating each step of the workflow (with a translator) without interruption. This allowed staff members to remain attentive and focused on each step. English literacy was minimal amongst these staff members so translators were crucial to the training. Translators were NIMPE staff fluent in both English and Vietnamese that had attended the NIMPE LAMP training. Although staff were able to grasp the techniques easily through demonstration, it was challenging to convey advice and ways to increase productivity.

The review conducted by the trainers at the completion of the training identified some potential impediments to the success of the training (**Table 3.2)**. The two main issues that were identified in the review were the representation of communes and language barriers.

Table 3.2: Key impediments identified during rural health care staff training

Impediment	Implications to training and study
Training of staff unlikely to directly perform or supervise LAMP	Difficult to focus training solely on key staff that would be participating in the study and ensure their proficiency
Study communes were not represented equally	Trained staff would have to teach other staff at home commune and their proficiency could not be assessed by trainers. Some study communes were operated by many trained individuals and some were run only by one trained individual
Language barriers prevented intricacies of LAMP being explained to staff	Staff did not have the ability to resolve any technical issues encountered

3.3.3 Challenges encountered in approaches for data entry

Epicollect 5 is a free web-based software platform with a smart phone application that allows users to create customised forms with various data entry options including dates, geospatial localisation data for mapping, images, and text. This can be tailored to local language requirements, including Vietnamese. These electronic data entry forms can be shared with many users, thus facilitating teams of Health Care Workers to deploy and undertake rapid population-based data collection activities. The data can be readily exported for analysis on other platforms. The initial intention of the study implementation was to have digital data entry for all aspects of the study. Early in the partner engagement process, it was apparent that Commune Health Care Workers and NIMPE staff were familiar with digital data entry processes using mobile phone apps. Therefore, this approach was able to be used for the initial household mapping survey (detailed in **Chapter 4**) and the passive case detection activities set up at the Commune Health Care Centres. This early partner engagement also determined however, that field staff were not as comfortable with a complete digital data entry process. This posed a major risk to implementation and was a potential threat to the quality of data that would be acquired. Therefore, it was decided that paper-based data collection would be undertaken for the cross-section survey that was used to collect samples for LAMP testing and to record the results of this testing at the Commune Health Care Centres (results detailed in **Chapter 4**). These data were then digitally entered using a separate Epicollect5 form. Examples of these forms can be found **Appendix 3**. Key limitations of data entry and the subsequent implications on data analysis are outlined in **Table 3.3.**

Table 3.3: Key limitations of using Epicollect for data entry

Limitation	Representation	Implication on data analysis
Drop down menus	Fields such as "LAMP result" having a drop down menu to select answers from, may result in more mistakes than if the result had to be manually entered instead	Accidentally choosing "negative" instead of "positive" affects prevalence estimates and downstream testing such as PCR testing
Duplicated and incomplete entries, and data entered in incorrect fields	Some entries had the same barcode (which guided all analysis) but different demographical data such as age or gender; some barcodes were not entered into the correct field	It was not possible to determine which demographic and testing data was correct from duplicate entries. Incomplete entries and entries without barcodes were excluded from analysis
Mistranslated fields	Fields to capture microscopy data were mistranslated resulting in staff uploading a photo of the microscopy slide rather than entering the result of the slide examination	Microscopy could not be adequately compared to LAMP

3.3.4 Logistical challenges

Procurement of equipment and reagents was one of the biggest logistical challenges of the study. This and other challenges are highlighted in **Table 3.4.** These challenges were largely unforeseen prior to the commencement of the study, and so study organisers had to manage situations to the best of their abilities with the resources available. The delays caused by these logistical challenges resulted in an overall delay to study commencement which in turn resulted in shorter durations spent at each study site. This may have impacted the amount of samples that field staff were able collect.

Table 3.4: Logistical challenges and their impact on the study

Limitation	Implications to study
Procuring specific scientific equipment with language barriers	Equipment such as heat blocks, centrifuges, and pipettes were difficult to obtain in Vietnam and so had to be brought from Australia increasing time delays and funding expenditure
Study sites could not receive equipment directly	All equipment was procured and delivered to NIMPE headquarters in Hanoi, then transported by air to study sites, increasing time delays and funding expenditure
Environmental factors could not be controlled when transporting samples	At the end of the study, DBS samples, remaining LAMP kits, and equipment were transported back to Hanoi. During this time, they were exposed to temperature and humidity fluctuations which may have compromised them for future testing
Unforeseen time delays	Delays in procurement and transporting equipment delayed the commencement of the study, the duration of the study, and how much time could be spent at each field site

3.3.5 Major themes highlighted from feedback survey

Successful implementation of a new technology like LAMP, and its long-term integration into a health system like the NMCP, is greatly influenced by end-user perceptions and user-experience of the technology. In order to assess this, in-depth interviews were conducted with three of the primary LAMP end-users to obtain insights into the following issues: theoretical understanding of LAMP; relative benefits and limitations of LAMP compared to other approaches; obtain feedback to the sampling and testing workflow so that improvements can be made, and to get the impression of end-users about the feasibility of this technology at this level of the health system. All three operators were NIMPE staff members, female, and aged 30 – 45 years old. One had a molecular background (Operator A), and the other two field epidemiological backgrounds (Operators B and C). Questions were based around the understanding of LAMP, limitations of LAMP, and their views on improving the implementation approach. A qualitative approach was used so we could capture the diversity and richness of the end-users experience and to minimise bias. It should be noted that the intended operators for this study and for future scale-up implementation, were commune health care staff but due to the time constraints of the project, NIMPE staff conducted most of the LAMP testing with the support of the commune health care staff.

Understanding of the principles of LAMP

Operators were asked about their understanding of LAMP principles. All operators understood that LAMP could detect the malaria parasite; however, the extent of understanding differed. Operator A possessed a detailed understanding of LAMP, albeit partially confusing LAMP with other molecular tests: *"LAMP reactions rely on isothermal conditions in which Taq (DNA polymerase) is activated… and when looped DNA is replicated, it is detected by the presence of magnesium phosphate which results in a colour change".* Operator B did not understand the principles of LAMP, and also did not think it was a necessity as long as they could physically perform the test. Operator C was more concerned about how the LAMP kits were developed, believing that having the reagents on the lid of tubes posed a contamination risk.

Advantages and limitations of LAMP

Operators were asked what they perceived to be the advantages and disadvantages of LAMP compared to other malaria diagnostics. Operator A explained that LAMP was more sensitive than microscopy and compared well to PCR, also adding: *"…it is easier than PCR and therefore you could potentially use it in the field. Additionally, the duration (of LAMP) is much shorter so I think it is very good".* Operator B, who is a trained microscopist, identified the time LAMP takes as a limitation, stating *"LAMP is a much longer process which achieves the same results as microscope slides. I can usually get a result with a slide in 2 minutes whereas with LAMP, it takes much longer. This may be a problem when you need to get results fast".* Conversely, Operator C believed LAMP was a very useful tool, however was worried about its predisposition to contamination saying, *"What I'm concerned/afraid about most is keeping the lab room clean and sterile to avoid contamination. The room needs to always be stable. This is only way that we will obtain accurate result".*

Impression of sample management tools

When asked about the sample management tools used in this study, the overall consensus was that operators found the barcode system to be cumbersome and time consuming;

Operator A said *"I don't like sticking on the codes...It is very annoying. In reality, sticking codes on the blood samples collected is okay. But when you are sitting down and working, you just need to put the samples in an order. It is much more effective, and you won't make mistakes".* Operator C stated *"...the step that wastes the most amount of time is the sticking on labels (bar codes) step. If you remove this labelling step, running LAMP would be much faster".*

Using Epicollect to report data was seen favourably by two operators *"I think it is very easy to use. For those entering data, and later for those compiling the data, it is more than okay"* (Operator A); and *"I think Epicollect is very easy to use. I think its only downfall is that it requires internet"* (Operator C). However Operator B found it tedious recalling *"Epicollect seems a little too prolix".*

Improving sample collection

Operators were asked how sample collection could be improved. They raised the importance of having incentives for participants and ensuring funding bodies were aware of this requirement.

"...all the villagers regardless of where they're from, only give blood if they're given incentives. If we have nothing to give, asking for their volunteering is something almost unheard of. We need cooperation between those who head these finances and ourselves. It would make sample collecting much easier". - Operator B

Operator C shared a conversation she had with some participants; "*They told us that they received money from other studies (as incentive). However, we did not have money. So, a lot of difficulties were encountered during sample collection"*. Operator A added that the day and time of collection was also extremely important; "(*sample collection) is dependent on the villager's job… the time they go to collect blood should be when the villagers are not out working, or on the weekend... I think the evening or the early morning would be good".* Another observation that operators made was that a designated sample collection point would make sample collection more convenient and would therefore result in more samples collected.

"To make it convenient for everyone, it would be good to have an announcement first to gather everyone...if everyone's in once place; we won't need to transport any of the sample collection equipment from house to house...especially because a lot people can see these things and they become very afraid and don't like it at all" - Operator B

Operators also expressed that the amount of blood required from each participant might be too much to obtain from a finger prick and that participants were not comfortable giving such as large donation.

"I think that the amount of blood collected is too much. A 400mm lancet…piercing a patient's finger with that is really quite painful. It needs to go quite deep into the skin to get blood. If you pierce it too shallowly, there is no way you can squeeze the required amount of blood out of the patient's finger...it would be good to get blood from the veins. Obviously, it would cost more and require more expertise…" - Operator B

"...sample collection was very hard because the villagers accused us of taking too much blood. We explained to them that we needed that much blood to do enough tests to detect the (malaria) parasites. Secondly, they were comparing our LAMP study with other studies." - Operator C

Feasibility of LAMP at health care level

The final part of the survey asked operators if they felt LAMP was practical at the commune level and if it would be possible for Commune Health Care Centre staff to carry out LAMP as part of their routine duties. They were unanimous in saying that with guidance, LAMP was simple enough for Commune Health Care Centre staff to perform with Operator A stating *"I think that if we train them well, they could perform this test. With the machinery and equipment we have invested in, I think that they could do it".*

Operator B added that although commune level staff are not laboratory trained, they could perform LAMP with enough training; *"... the main issue about the health care centre staff, is they come from diverse areas. Some are not that skilful in lab work – most are just nurses. We've only just got doctors here and even they may not be skilled in the lab. Most of these doctors are young and inexperienced. But I believe if they had guidance, they would be able to do it (perform LAMP). The staff here are usually trained to do more than one job... (LAMP is) equal practicality as microscope slides".*

3.4 Discussion

This malaria elimination pilot study highlighted a range of important factors to be considered if a new diagnostic tool, such as LAMP, is to be sustainably integrated into a health system, especially in a resource-constrained setting. The project provided an opportunity to introduce this new technology into the NMCP of Vietnam at a national, provincial and commune level. Key findings from this work are outlined in **Table 3.5** and are discussed within the context of an assessment that was recently conducted with the NMCP of Vietnam. The 2018 assessment conducted by the WHO and NIMPE identified areas in which provinces were meeting guidelines, and areas where improvements could be made. This included an assessment of case management, surveillance and response, and vector control. The NMCP review involved face-to-face interviews, reviews of documentation, site visits, and data analysis. A range of stakeholders were approached including those in planning, technical partners, the public sector, and target communities at all levels of the health care system [86].

The findings from this study, complement WHO's Framework for Strengthening Health Systems [87]. This framework consists of six building blocks; (1) Service Delivery, (2) Health Workforce, (3) Information, (4) Medical Products, Vaccines, and Technologies, (5) Financing, and (6) Leadership and Governance. **Table 3.5** addresses Service Delivery and Information, by identifying that reporting systems are not uniform across Vietnam, and that resource and logistical requirements are challenging in rural settings and with migrant populations. Health Workforce is characterised by needing to target an intervention at the right level of a health care system, ensuring acceptance and promoting education and training of specialised skills at all levels, and Medical Products, Vaccines, and Technologies are assessed as a whole throughout this study. Finance and Leadership and Governance is also addressed as shown when the NMCP Review found political commitment was dependent on the level of support received. **Table 3.5** also further expands on this framework by addressing the need for implementation strategies to reflect current policy and highlighting that cultural differences and unclear communication can be severe barriers.

Table 3.5: Overview of challenges faced when implementing a new technology in resource-constrained settings

Area of challenge to successful implementation	Overview of considerations	Implementing LAMP in Vietnam
Policy	What the current policy is in-country and how this aligns with the new test or method to be implemented	The National Strategy for Control and Elimination policy bears no mention to asymptomatic carriers or high sensitivity molecular testing
Health systems	Dynamics within the hierarchy of staff in the health care system may be complex and may impact implementation if not targeted to the right level	Many tiers to health system, and study of new test would need to be evaluated and accepted by each level
	Data reporting and management systems may already be in place so new methods should be compatible with these systems	Malaria Information System and Health Management Information System in place and each Global Fund project also uses different protocols
Acceptability	The new technology should be accepted and understood at all levels of the health care system to ensure sustained implementation	Higher levels of health system have a more thorough understanding of technique, its purpose, and the global goals of malaria than staff at lower levels
Logistics and resources	If the technology is to be implemented in remote areas, the following need consideration: how resources are to be delivered, how samples will be taken, how best to convey results to patients	Internet access and transport are generally reliable at all health system levels, but refrigeration and follow up of migrant populations are limited
Language and communication	Differences in language and cultural beliefs may be apparent between locals and implementers and they may affect conveyance of knowledge and adherence to protocols	English proficiency is low at most health system levels making explaining complex technical procedures challenging
Education and training	Depending on the level in the health system the technology is to be implemented, experience and technical skill may be limited so the training of technology should be modified accordingly	Technical knowledge and skills in molecular techniques are limited at all health system levels

3.4.1 Implementing new technology should be supported by policy, but also has the ability to inform new policy

Successful and sustained implementation of all diagnostic, therapeutic and preventive tools, within the context of regular NMCP activities, requires support from the relevant national health policies. Therefore, before implementing new tools, there needs to be careful evaluation of existing country policies. In the case of new technologies, like LAMP, it is likely that there are no existing policies specific for the technology. If this is the case, then policies need to be examined to determine if the issue that the technology seeks to address is supported in the broad terms of the policy and objectives of the program (e.g. the need to identify and treat asymptomatically infected individuals). At the time of this study, Vietnam's *National Strategy for Control and Elimination of Malaria* policy (2011) did not have any guidelines for the management of asymptomatically infected individuals, nor did it have clear policy on high sensitivity molecular testing for this application. This absence in national policy likely reflects the evolutionary process that policies take as NMCPs shift from disease control to diseases elimination, and the different strategies, practices and tools that are required for these approaches. In the absence of national policy to guide the NMCP, regional and international policies can be very useful in assisting to engage with national stakeholders, and to assess the scientific evidence and implementation research required to change policy. The WHO's *Strategy for Malaria Elimination in the Greater Mekong Subregion* (2015) describes detecting and treating asymptomatic cases as a key objective in eliminating *P. falciparum* malaria. An important part of this process with new technologies is to assess their local relevance and to determine effective ways that they may be applied. This also includes a process to engage with all relevant stakeholders, national and local, to understand the technology, and assess its advantages and limitations relative to other alternatives. Failure to undertake locally-relevant research and engagement processes on new technologies, may lead to a lack of awareness of these options, their potential impact, and may decrease the likelihood that a pilot study attempting to address these needs are accepted and funded.

In addition to policy itself, the establishment of practical guidelines and adherence to recommended practice are also critical in determining the effectiveness of policies. In the NMCP review mentioned

above, adherence to general malaria-related policies was found to vary considerably between provinces in Vietnam. In some provinces, NMCP guidelines were followed exactly, but in others practices were adapted to suit local circumstances [86]. The review also uncovered a disconnect between what was noted at the national level and what was actually implemented at local levels. This indicates that perhaps policy may not be well informed by current research or that policy is not being followed at the local level. Undertaking implementation research, such as the study presented in this chapter, needs to consider the pathways that the research findings can take to influence stakeholders and the processes of impacting on policy.

3.4.2 All levels of the health system should be engaged to promote implementation of a new technology

The health care system of Vietnam consists of the national, provincial, district, commune, and village levels. Each level is represented by individuals of different education and experience levels, and thus different levels of knowledge of malaria in the global context [86]. Navigating the various levels of a health care system can be challenging, especially when proposing new trials to stakeholders in the hope they will engage in the trial. One way to decrease resistance to new trials is to provide further education and training opportunities to staff at all levels. In Vietnam, this was common practise with many provincial and district level health care staff attending the training sessions of this study. However, it may be beneficial to have separate training sessions for these individuals to demonstrate the theoretical principles of the new test and the potential outcomes, without utilising limited resources to train them in techniques they would not likely perform themselves.

The NMCP Review found political commitment in Vietnam to vary between levels of the health care system according to the level of support provided. In some areas political commitment at the provincial level seemed to be relatively low due to low budget allocation for malaria-control activities, but were higher at the District level where the People's Committee were actively involved [86]. Implementing a new tool can be difficult in areas where political commitment is low, therefore this should be assessed

prior to commencing a field study. Field sites for initial implementation studies may be more successful if conducted in areas with high levels of support.

A major limitation of this study was inaccurate data recording. Many discrepancies were found after the conclusion of the study, such as duplicate and incomplete entries, and data input into incorrect fields. The smart phone application platform of Epicollect was a convenient and efficient way to enter data, especially when data was being entered by multiple operators, however it may be prone to operator error. A simple solution might be to use tablets instead of smart phones (a bigger display may make it less likely to select the wrong option), or not including multiple choice or dropdown style options (manually entering data rather than selecting from a drop down menu would eliminate selecting the incorrect option). The paper forms completed during sample collection were presumed to be correct as participants were present (i.e. could double check their information) and more time was taken to manually complete each field. A platform that could scan these paper forms and generate a data set from the scanned documents would be ideal. This method would also mitigate extra work for health care staff as they would not have to enter data on multiple mediums. Such platforms do exist (e.g. Wondershare, Docparser), but a license must be purchased and health care centres would need to have internet access and scanning facilities. Many similar platforms to Epicollect exist, but few are open source and cloud based. Epicollect is a valuable tool but precautions should be taken to ensure forms are designed to account for subtle mistakes made during data entry, such as selecting the wrong option in a drop-down menu.

In Vietnam, several data reporting systems have been rolled out, but many of these have encountered challenges in implementation and have therefore had limited effectiveness. These systems include the Malaria Information System, the Health Management Information System, and the electronic Communicable Diseases System. According to the NMCP Review, most of these systems are not fully functioning effectively due to server update requirements; or are not linked to each other, resulting in the duplication of much information. Staff at all levels are over burdened with the reporting requirements associated with Global Fund grants and other disease-specific programs and are therefore

unable to enter data into these systems regularly as a result [86]. As observed in this study, adding further data entry requirements into the workload of health care workers can result in data entry mistakes and missing data. To maximise the likelihood that data will be captured accurately, future studies may be able to utilise existing data entry systems where possible to reduce the workload of staff. It should be noted however that the requirements for elimination programs will differ to those of control programs, and so separate systems may be required.

3.4.3 Acceptability of the new technology is critical at all levels of the health system

Acceptability is a key factor in determining whether a new technique will be successfully implemented into a health system [88, 89]. Staff acceptance at all levels of the health care system is important. Higher levels (national and provincial) must accept a new test before it can be incorporated into policy and used at the lower levels. Staff acceptance is also important at lower levels of communes and villages because this is where the main workload for the implementation of new technologies will lie. These end users need to manage the workflow of new technologies and intensified malaria elimination activities alongside their other duties. Finally, the community needs to accept a new technique as their cooperation is essential to obtaining accurate results. Input from the community also provides an important opportunity to learn how the technology can be improved. Without acceptability at all levels, a new test is less likely to be successfully established so methods to specifically address this issue should be incorporated in study designs.

Studies have shown that acceptance increases over time as understanding of new interventions improves [90]. Stakeholders may be reluctant to commit to new technologies until they start seeing results from local field trials [90]. This study did undertake a broad assessment of staff acceptability of LAMP. With the limited scope possible in this study, the end-user assessment indicated potential limitations in the understanding of LAMP principles, of result interpretation, and a suggestion that staff may have been concerned about the sustainability of the technology. This indicates that this type of technology may need further training and support if successful widespread implementation is to be successful. NIMPE and field staff were able to easily perform LAMP testing once trained, but may have benefitted from more training in the principles of LAMP so that they were equipped to troubleshoot any issues faced.

Dissemination of most study findings are also limited to scientific journals which are unavailable to commune and village level health care staff. Sustainability was not considered as a major factor in this pilot study of the technology. The potential for this technology is encouraging (**Chapter 4**) and therefore raises questions of how it could be introduced in an affordable and sustainable manner. This will require ensuring an adequate understanding of the technology at all levels of the health care system, dissemination of findings from the study in a format that is accessible to all relevant stakeholders.

3.4.4 Logistics and resource related challenges can hinder successful implementation of a new technology

Logistics and resource related problems can impact on the success of field studies and programmatic implementation. These issues may be even more apparent when implementing new technologies, because the equipment and logistic systems are yet to be established and tested under local conditions. This study uncovered the increased costs and time delays that can easily result from logistical problems. Most studies are time constrained due to a location or staff members having limited availability. In malaria studies, extra complexity is added with seasonal patterns of transmission. Given the predisposed time restrictions, it is practical to minimise or control for any additional time delays.

The NMCP review found that financial resource stability requires strengthening in some Provinces. Many Districts are financially dependent on the Global Fund for Malaria related budgets as commune level funding is capped for each Province and is generally utilized for salaries [86]. As with time, field studies are often constrained in financial support and thus care should be taken to avoid additional expenditure.

Extensive planning prior to the study should investigate how to mitigate these factors so they do not impact the study.

Working with transient or hard to reach populations presents an unavoidable logistical challenge to elimination activities. Not only are these people difficult to reach and test, they are also difficult to follow-up. If study outcomes are dependent on transient populations, approaches that aid in determining their location quickly, safely and reliably are required. It may be possible to engage with these

populations directly and promote an ongoing collaboration where they can maintain their lifestyle but also contribute to study findings. It may also be pertinent to engage with other sectors such as the military that have more reliable access to these populations. These individuals are important in elimination and thus sufficient planning and resources should be dedicated to securing their inclusion studies.

3.4.5 Understanding language and cultural differences are essential in implementing a new technology

This study highlighted the importance of understanding the challenges posed by language barriers and cultural differences. While inherent to any field setting in a non-English speaking country, they are obstacles that must be overcome if an intervention is to be implemented, sustained, and address the intended problem. High quality translation is essential, especially for scientific terminology.

Understanding the way a studied society functions is important. Studies are often designed prior to being in country; in this instance all SOPs, sampling methods, and data collection protocols were initially developed based solely on the study implementers' past experiences. If possible, field staff should be actively engaged in the design of the intended protocols, as they are the most equipped to determine if protocols will be feasible and accepted by the community. Using sample tracking as an example, if field staff were actively involved in the designing of participant forms, the forms may have been more balanced in capturing all the necessary study data points, while not increasing the workload of staff too much (i.e. not including questions where the answer could be inferred from other data points, or finding an alternative to the barcoding system that field staff found cumbersome to use). This may have improved data collection, as forms would have been shorter and easier for field staff to manage with their existing routines. Language barriers and cultural differences are often unavoidable when implementing a new technology in malaria endemic countries. However they need not hinder a study if these differences are accepted and used to tailor the study in accordance to the cultural norms of that population. Including representatives from all those involved in the study in the planning process, will allow for study outcomes and workloads that are suitable for everyone.

3.4.6 Education and training should be tailored to suit each level of the health care system

Equally important in understanding cultural differences and amending protocols to suit, is educating field staff beyond the protocols themselves. It is important to ensure a thorough understanding of how to perform a test, but understanding the reasons behind certain processes is also beneficial. Using sample management as an example again, if the purpose for each data field was explained to staff, perhaps they would be more likely to precisely complete forms. If they understood that accurately acquiring data such as a participant's age, gender, home location, and previous history of malaria could provide valuable insights into disease transmission and risk factors, then they may be extra vigilant when reporting on those fields. Similarly if emphasis was placed on comparing LAMP to other diagnostic tests, field staff may have been more likely to report microscopy results accurately and without bias knowing that the results could evaluate whether LAMP is more sensitive than current screening methods (operators were observed on occasion to re-examine slides they evaluated as negative when they learned that the LAMP result for that same sample was positive). Studies have shown a desire of field staff to increase their knowledge in areas such as malaria prevention, field work coordination, and implementation of specific public health activities; and report field staff receiving insufficient remuneration and acknowledgment [91] Another study surveying Health Care Workers in high endemic countries highlighted recurring challenges to be financial instability, ever changing job descriptions, and limited malaria knowledge [92]. These studies demonstrate that workers regard learning about the tests they are using, and understanding malaria more broadly, as highly as they regard financial stability.

Thorough training was paramount in this study, as most techniques used were new to field staff. The numerous delays from ethics application requirements, collaboration challenges, and procurement errors decreased the amount of time that could be dedicated to training. Training was not conducted in a manner typical for Vietnamese staff, so they had to learn a new and complex technique in a teaching style different to their own. It would have been advantageous for trainers to be informed on the preferred training style, enabling trainers to tailor their teaching style so that it was more easily comprehendible by Vietnamese staff. The NMCP review found that training materials of RAI-funded projects were too complicated and not suited to the needs of Commune Health Care Worker and Village Health Care

Workers [86]. This may impact the ability of staff to fully understand the training materials as well as impacting the motivation and adherence of staff in performing the new technique. Specifically tailoring training sessions and associated materials to the individuals being trained should be a key consideration in integrating a new test into routine health care.

Exclusively training key staff is vital when time and training resources are scarce. In this study, training sessions were attended by both field staff and managerial staff. This expended valuable resources and time as higher level staff did not directly participate in this study. It may be more practical for separate training sessions to be held. Managerial staff can attend training sessions based on theoretical practises and field staff can attend focused practical training sessions. This ensures enough time is dedicated to both groups and training sessions are more focused on the specific needs of each group. Similarly, although Commune Health Care Worker were trained, it was apparent at the conclusion of the study that NIMPE staff performed the majority of LAMP testing, with Commune Health Care Workers only assisting in sample collection, if at all. It is not known why this occurred; Commune Health Care Workers may have been too busy with their routine duties, or perhaps there was a miscommunication with NIMPE during the study design phase. Future studies should first establish which staff will directly participate in the study, which staff will be responsible for carrying out the test should it be integrated into routine duties, and those individuals should be given priority in training while all other levels receive tailored and less extensive training as required.

3.5 Conclusion

This pilot study highlighted the importance of quality education and training, identified issues in conveying scientific processes across language barriers, and emphasized accurate data reporting. Feedback from operators provided insight into the perceived utility of LAMP and highlighted improvements for sample collection. In order to successfully implement a new tool and increase the likelihood that it will be integrated as common practise, the dynamics of the health system must be carefully considered, the technique must be accepted at all levels in that health care system, and challenges in resources and logistics must be accounted for in planning.

4 Field evaluation of LAMP assay for detection of asymptomatic malaria carriage in Vietnam

4.1 Introduction

Finding and treating asymptomatically infected individuals poses a major public health challenge for NMCPs, but it is likely to be an important strategy to fast-track malaria elimination [79, 93]. Many factors need to be considered when screening for asymptomatic infections, with two important factors being sampling approach and the diagnostic test used.

To effectively assess the risk and burden of asymptomatic infections within a community (e.g. the border Provinces of Vietnam discussed in **Chapter 3**), a cross-sectional survey is likely to provide the most effective study design. This study design allows a large proportion of the community to be sampled over a short period and can give important insights into the prevalence of asymptomatic infection, identify the subgroups in the community that have higher risk of infection, and which can then be targeted with disease elimination strategies. In a malaria elimination setting, such as the low transmission seen in Vietnam, there is likely to be a low prevalence of infection, which then necessitates a large sample size in order to find enough positive samples.

As previously mentioned, highly sensitive molecular techniques have great potential in the detection of asymptomatic malaria infections. Two of the most widely used techniques are LAMP and PCR. LAMP has been recommended as the preferred technology to test large amounts of people with little resources and infrastructure [76, 94-96]. There have been relatively few field-studies assessing LAMP implementation [97-99], so the cross sectional study presented in this chapter also afforded an insight into how effectively LAMP might be applied for mass screening. The outcomes from this type of approach would be informative for identifying the best approaches for the NMCP to implement a national program of intensified screening and treatment. This would be especially useful in determining whether a MSAT, FSAT, or active case detection approaches would be sustainable and effective. Similar constraints observed in this study (i.e. time, finances, and logistics) would be encountered using these approaches in a national program using LAMP for high-sensitivity screening.

Although there is a rapidly expanding interest in geospatial mapping of malaria cases, there has been little done in combining these approaches with high-sensitivity testing that is conducted locally and

rapidly after sampling large populations. The use of geospatial mapping adds another level of precision to accurate identification and localisation of asymptomatically infected individuals. It can be used to determine which communities are more at risk than others, and can also be used to monitor the effect that any newly introduced interventions are having [100-102].

In this study, LAMP was used to detect asymptomatic malaria infection in rural Vietnam. The prevalence of asymptomatic infections detected by LAMP were compared to those detected by microscopy and PCR. GIS mapping was also used to chart asymptomatic infections detected by LAMP to determine if any relationships were apparent with interventions or with clinical cases.

4.2 Methods

4.2.1 GIS mapping to identify potential trends in malaria transmission

Prior to the LAMP study, households were mapped at each of the five communes by NIMPE staff with GPS-enabled mobile phones. Staff used a separate Epicollect form to record these results which included the commune, the village, the number assigned to that particular household, and the precise GPS location of the household. After geo-locating all households, NIMPE staff obtained data on malaria prevention strategies that were in place in each household (e.g. use of insecticide-impregnated bed nets, indoor residual spraying, etc). These data were again reported through an Epicollect form. StataSE (version 13) was used to clean the original dataset from Epicollect and QGIS (version 2.18) was used for all mapping activities.

For household survey, intervention, and clinical cases data

Cleaned data sets were imported to QGIS. Geospatial cleaning was performed within QGIS (where geospatial information was available) and incorrect coordinates were corrected. Data points that did not have geospatial information were allocated to a geocentric location in the middle of the reported village. Topography maps were obtained from Google Earth and geographical maps were obtained using

Humanitarian Data Exchange (version 1.33.1). The Heatmap Styles tool of QGIS was used to create hotspots of clinical cases. These data were able to be mapped to the household level.

For LAMP data

Cleaned datasets were imported to QGIS. GPS co-ordinates were not collected for LAMP cases because the recruitment of the study participants was done as a cross-sectional survey in which people came to the village centre. Accordingly, the geospatial data was only resolved to village level. The Convex Hull tool of QGIS was used to create polygons of village boundaries by mapping external points.

4.2.2 LAMP detection

The commercially available Eiken kit was used to screen asymptomatic individuals in five communes as part for the RAI study. Detailed protocols of this study are described in **Section 2.1** and detailed protocols for LAMP testing are described in **Section 2.1.2**.

4.2.3 Confirmatory PCR testing of LAMP positive samples

Selection process of DBS subset for PCR testing

As previously described (**Chapter 2**), DBS samples were collected from all participants and were transported to Australia. Due to time constraints and limited funds, a subset of 500 samples were chosen for analysis. The sample barcode was used to search the Epicollect database to determine commune and LAMP result. Some barcodes could not be matched to Epicollect, so those samples were excluded from further analysis. Once organized into commune, all LAMP positive cases were tested for malaria infection by PCR. Additionally, one-third of negatives from the same commune were randomly selected for PCR analysis. This was true for all communes except Dak Ngo, as no DBS samples were transported from this commune. Additional samples were chosen at random to reach the 500 sample target. This selection process is depicted in **Figure 4.10**.

Validation of LAMP using PCR

LAMP testing was conducted in the field at the time of the study, using whole blood from a microtainer sample. LAMP results were obtained as a visually-interpreted end-point read from the commercially-available Eiken *Plasmodium spp*. test. The PCR was conducted on DBS at Burnet Institute in Melbourne

approximately 9 months after the sampling period. PCR testing was first conducted using a *Plasmodium* genus PCR assay with a 18S rRNA target that is highly conserved in all human *Plasmodium* species. The samples that were PCR genus positive were then tested with *P. falciparum* and *P. vivax* specific 18S rRNA targeting assays to determine species. The PCR was interpreted as a real-time amplification curve from a QuantStudio instrument. PCR results were analysed as continuous data (i.e. cycle threshold values) and categorical data (defined by negative controls determined thresholds).

4.3 Results

4.3.1 Using GIS mapping to plot household locations, interventions in place, and clinical cases participants

Distribution map generated from house hold survey

Over the course of 3 weeks, teams visited each of the five communes in Binh Phuoc and Dak Nong Provinces, collecting GPS data on mobile devices at the household level. Approximately 11,000 houses were mapped during this period, and this makes up the base layer of further mapping activities (**Figure 4.1**). Blue points represent households, grey lines represent commune boundaries and orange lines represent main roads. The households were then overlayed over the forest coverage; here pink points represent household and black lines represent commune boundaries (**Figure 4.2**).

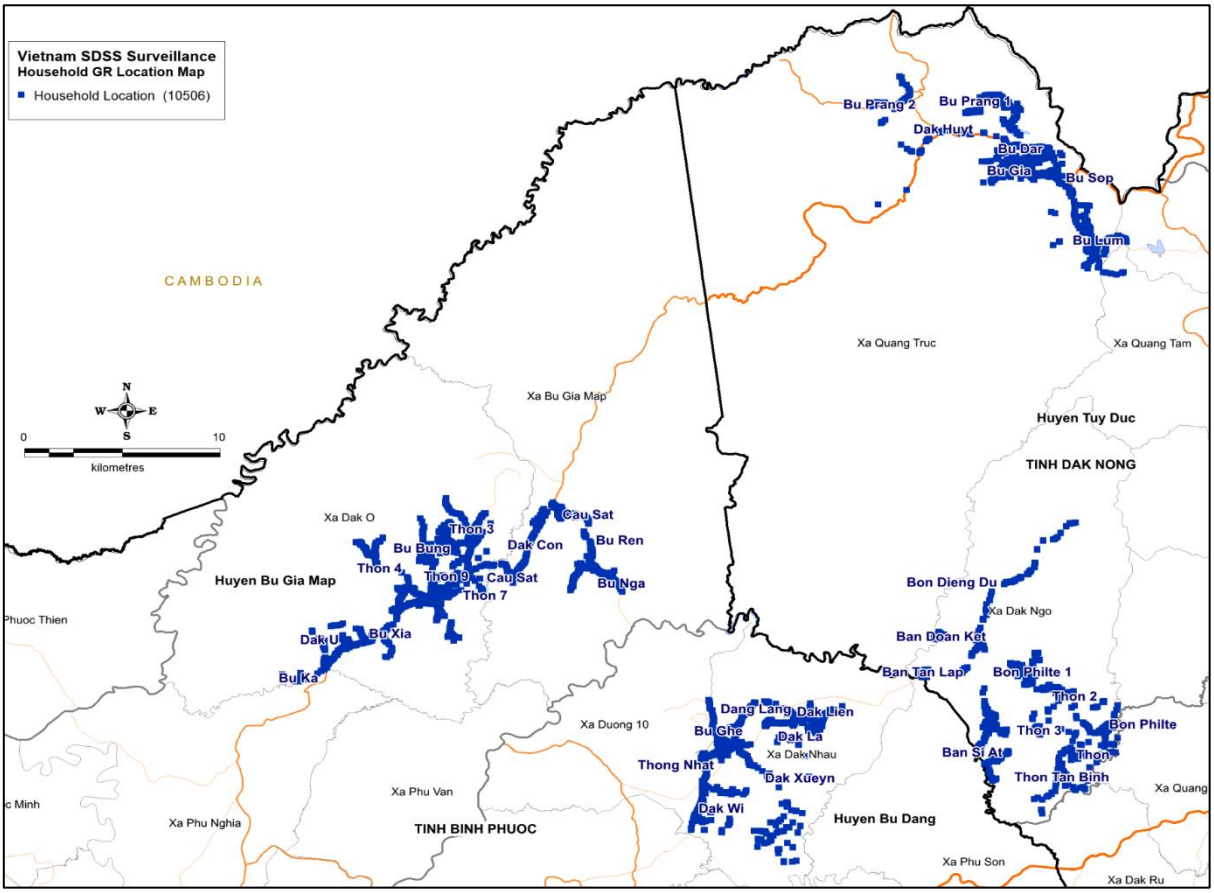

Figure 4.1: Base layer of household mapping survey

Approximately 11,000 households and 45,000 people were surveyed. Each blue marker represents the GPS location of a household, grey lines depict Commune borders and orange lines show major roads. Epicollect mobile application was used to capture GPS location, StataSE was used to clean data sets, and QGIS was used to develop map. Map developed by Dr Ricardo Ataide of the Burnet Institute

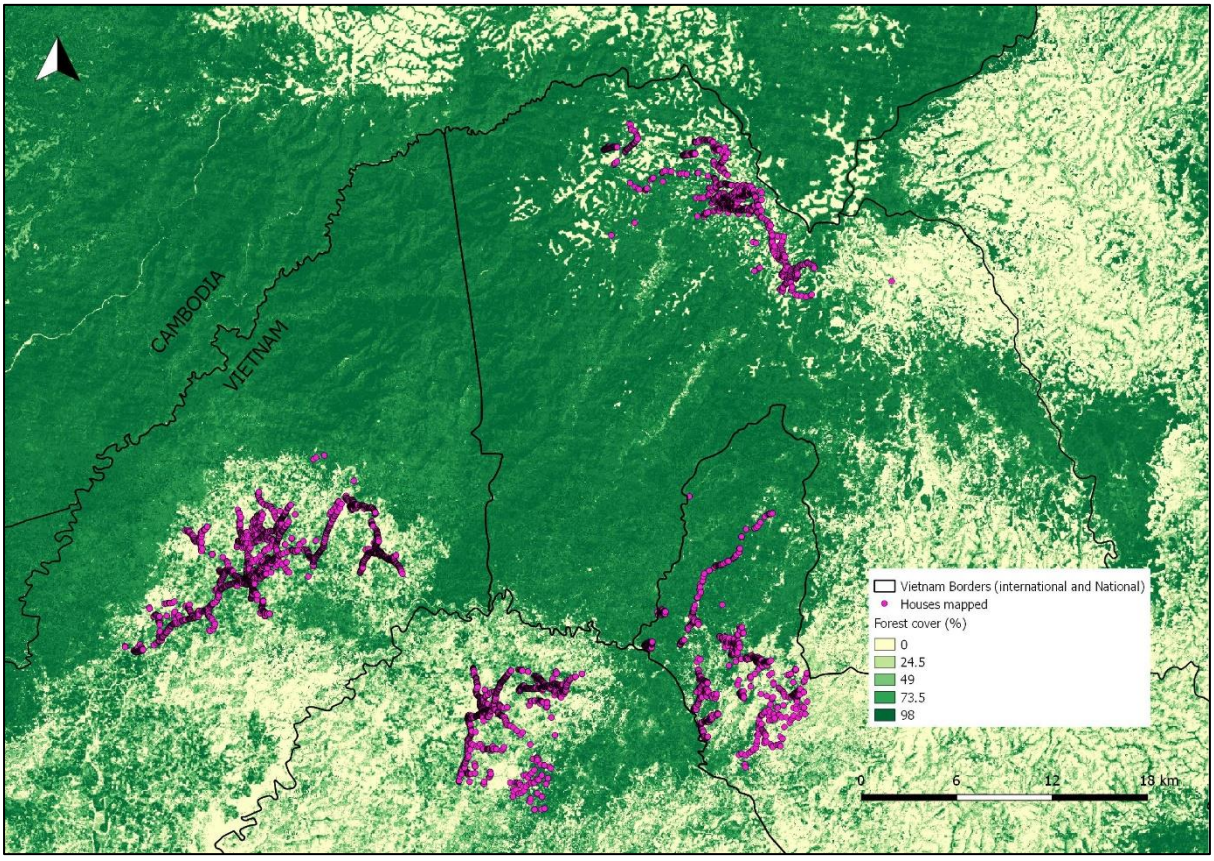

Figure 4.2: Forest cover overlayed on household survey map

The base layer of the household map (previous figure) overlayed onto forest cover map. Each pink marker represents the GPS location of a household and black lines depict Commune borders. Intensity of green indicates percentage of forest coverage. StataSE was used to clean data sets and QGIS was used to develop map. Map developed by Dr Ricardo Ataide of the Burnet Institute

Interventions in place in study communes

Intervention variation between communes in the same Province can be clearly seen in **Figure 4.3**, as can variation between villages of the same commune. In some communes, most villages applied a homogeneous selection of interventions as seen in Quang Truc and Dak Nhau (large blue rectangles), which appeared to predominantly use a combination of both IRS and ITN (blue circles). In other communes a heterogeneous mix of all interventions seemed to be in use like in Bu Gia Map and Dak O (indicated by a mix of orange, yellow, blue and red circles). Neither IRS nor ITN use was reported in most households in Dak Ngo (large red rectangle). The use of a malaria interventions often differed even between households within the same village, where a cluster of houses might have both interventions in place and a neighbour cluster of houses would have neither, (e.g. Dak O- large green circle). All subsequent mapping will focus on Dak O and Bu Gia Map communes as they had the more diverse intervention strategies in place.

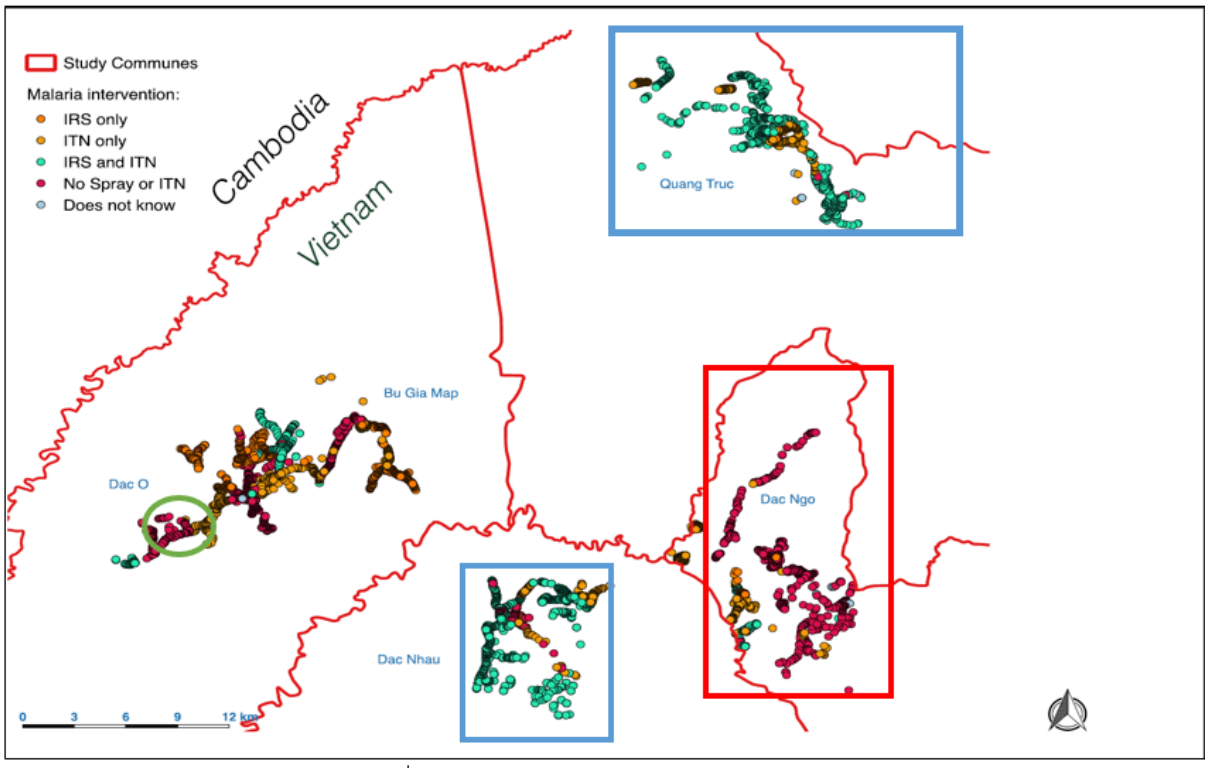

Figure 4.3: Intervention type and coverage difference was heterogeneous between different communes and between villages in the same commune.

Interventions were compared across communes. Interventions differed at the commune level as seen in Quang Truc which predominantly used IRS and ITN (blue rectangles), while Dak Ngo largely lacked any interventions (red rectangle). Interventions also differed between villages within the same commune. For example, one village in Dak O had no interventions in place (green circle) while neighboring villages on either side had ITN and or IRS interventions. Orange dots indicated IRS use, yellow dots indicate ITN use, green dots represent both IRS and ITN use, and red dots represent no intervention in use. Red lines represent commune borders. Epicollect mobile application was used to capture GPS location, StataSE was used to clean data sets, and QGIS was used to develop map. Map developed by Dr Ricardo Ataide of the Burnet Institute

Distribution of clinical cases reported by NMCP

Reported clinical cases (symptomatic patients that presented to the Commune Health Centre in Dak O and Bu Gia Map communes were overlayed on the household survey map (**Figure 4.4**). Cases are represented by stars, with *Plasmodium* spp (species unspecified) in white, *P. falciparum* in red, *P. malariae* in green, and *P. vivax* in yellow. A variety of species appear to be prevalent in this area and infections can appear in clusters, but also as isolated events. *P. falciparum* infections appeared to be most prevalent, followed by *P. vivax*, and *P. malariae*. It is not known why some cases were only reported as *Plasmodium* spp. Infections were detected by either microscopy or RDT.

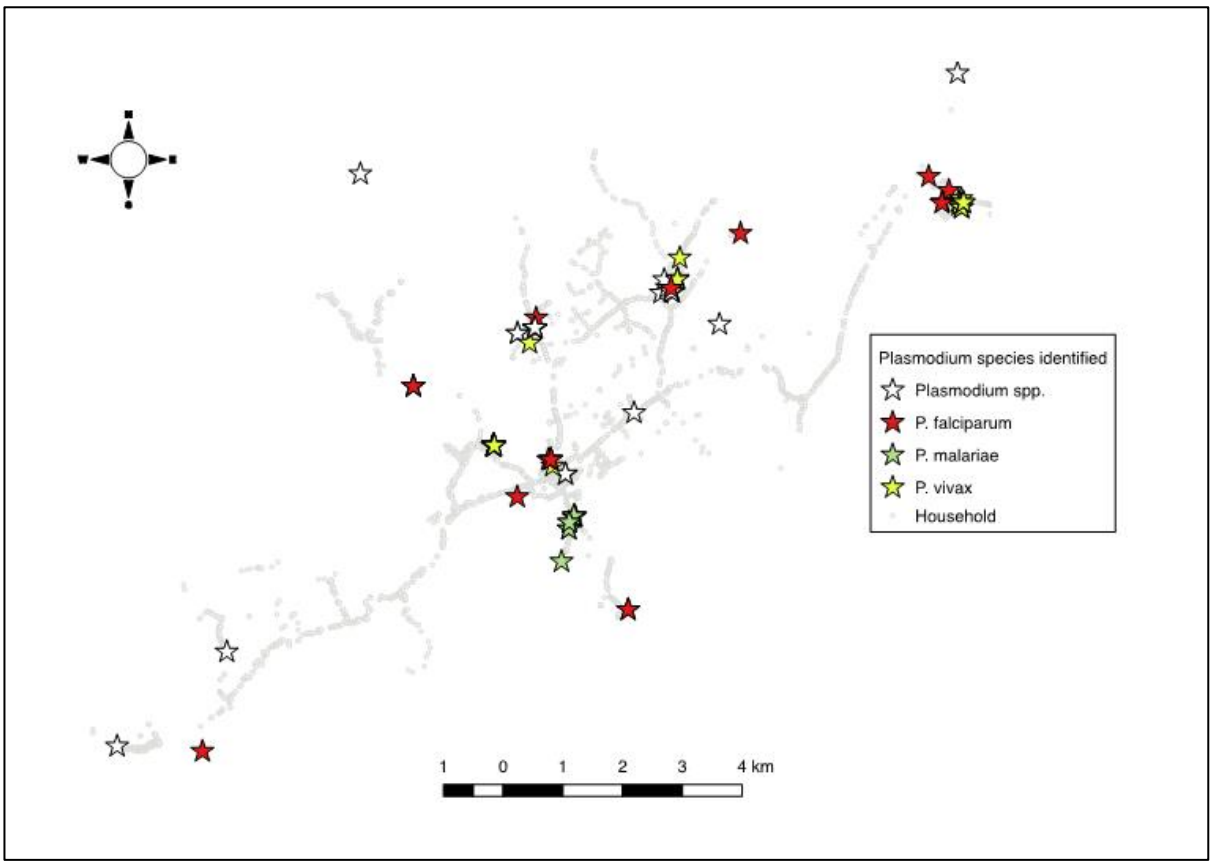

Figure 4.4: Symptomatic cases present in Dak O and Bu Gia Map communes.
Clinical cases were overlayed with household map. Grey dots represent individual households and stars represent reported cases. Red stars are used to represent *P. falciparum* cases, green for *P. malariae*, and yellow for *P. vivax*. In instances where a species was not identified, white stars were used to represent *Plasmodium* spp infections. Map developed by Dr Ricardo Ataide of the Burnet Institute

Demographics of participants surveyed with LAMP

A cross sectional survey was conducted across the five communes. This included recruitment of individuals of all ages. Participants attended the village centre for enrolment, informed consent, data acquisition and blood sampling. The basic demographics of enrolled participants can be found in **Table 4.1** below. These data were extracted from the Epicollect database. After removal of duplicated entries (n=303), entries with missing data (n=54), and entries with conflicting data (n=20), there were 5,421 enrolments across the five study sites (**Figure 4.5**). Over 1000 participants were enrolled from four of the five study sites; however, only 175 participants were enrolled at Dak Ngo. LAMP testing was not performed at Dak Ngo but these samples were collected and transferred to Quang Truc for processing.

Table 4.1: Basic demographic details of surveyed participants.

Total sampled		
	Bu Gia Map	n=1415
	Dak Nhau	n=1100
	Dak O	n=1364
	Quang Truc	n=1367
	Dak Ngo	n=175
Age (overall)*	median (range)	24 years (1-97 years)
Gender (overall)*	female	n=3132 (54.4%)

* Some participants had incomplete data sets and thus were excluded from this analysis

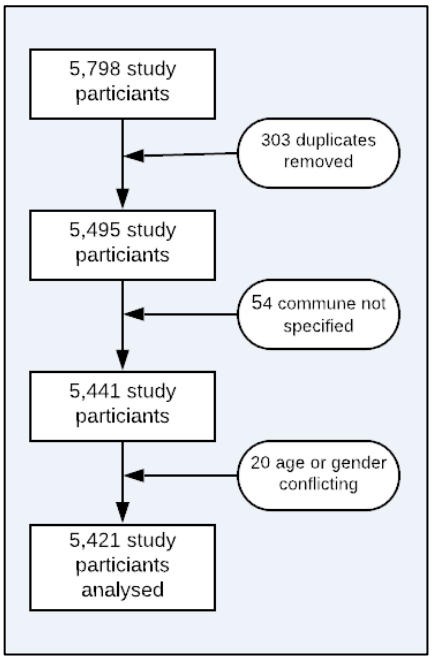

Figure 4.5: Exclusion process prior to analysing enrolments.
Epicollect data set was cleaned before analysis. Of the 5,798 data points entered into Epicollect, 377 were omitted from analysis due to being duplicated, missing information, or having conflicting information.

4.3.2 LAMP positivity rates compared to reported clinical cases and microscopy

Identification of an asymptomatic reservoir

Figure 4.6 shows the proportion of LAMP positive cases of all participants sampled in each commune. The greatest number of samples were collected from Bu Gia Map and the fewest from Dak Ngo. Positivity rates varied between communes ranging from 3.25% in Bu Gia Map and 0.18% in Dak Nhau. This corresponds to the proximity of these communes to the forest, which is the most likely source of mosquito transmission (**Figure 4.2**). Households in Bu Gia Map mostly had bed nets or indoor residual spraying, but not the combination of both, whereas Dak Nhau households mostly had both interventions deployed.

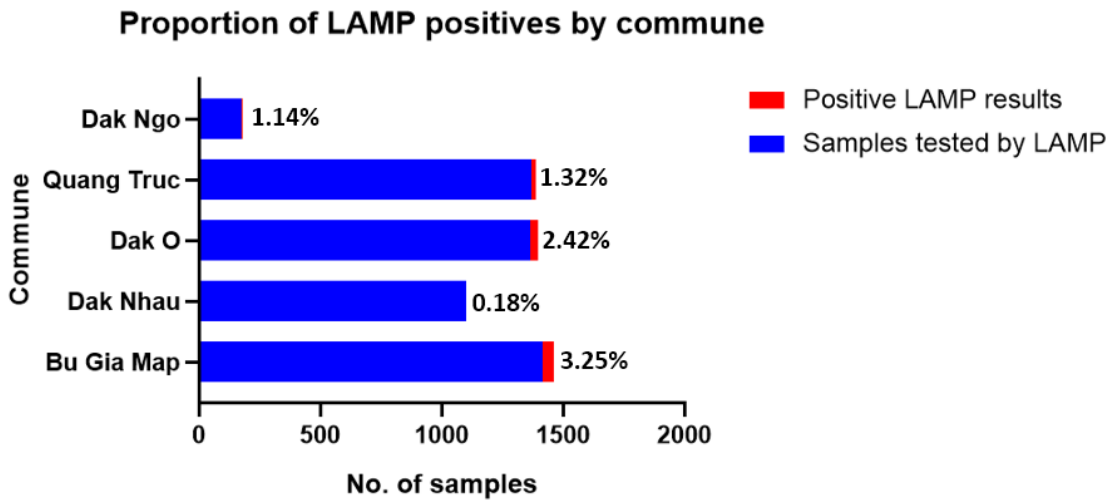

Figure 4.6: A substantial asymptomatic reservoir was detected using LAMP
101 asymptomatically infected individuals were detected from 5,421 people tested using LAMP (1.9% detection rate) varying between 0.18% and 3.25%, depending on the Commune.

Demographic comparison of clinical cases and asymptomatic LAMP positives

When comparing the number of symptomatic malaria cases (presenting at the Commune Health Care Centre) with the number of asymptomatic LAMP positive individuals reported over the same period, similarities in gender and age were observed. The majority of cases in both symptomatic and asymptomatic data sets were reported to be male (80% and 78% respectively) and peak incidence of symptomatic cases was seen in the age group of 15-25 years and between 10-20 years of age for asymptomatic cases (**Figure 4.7**).

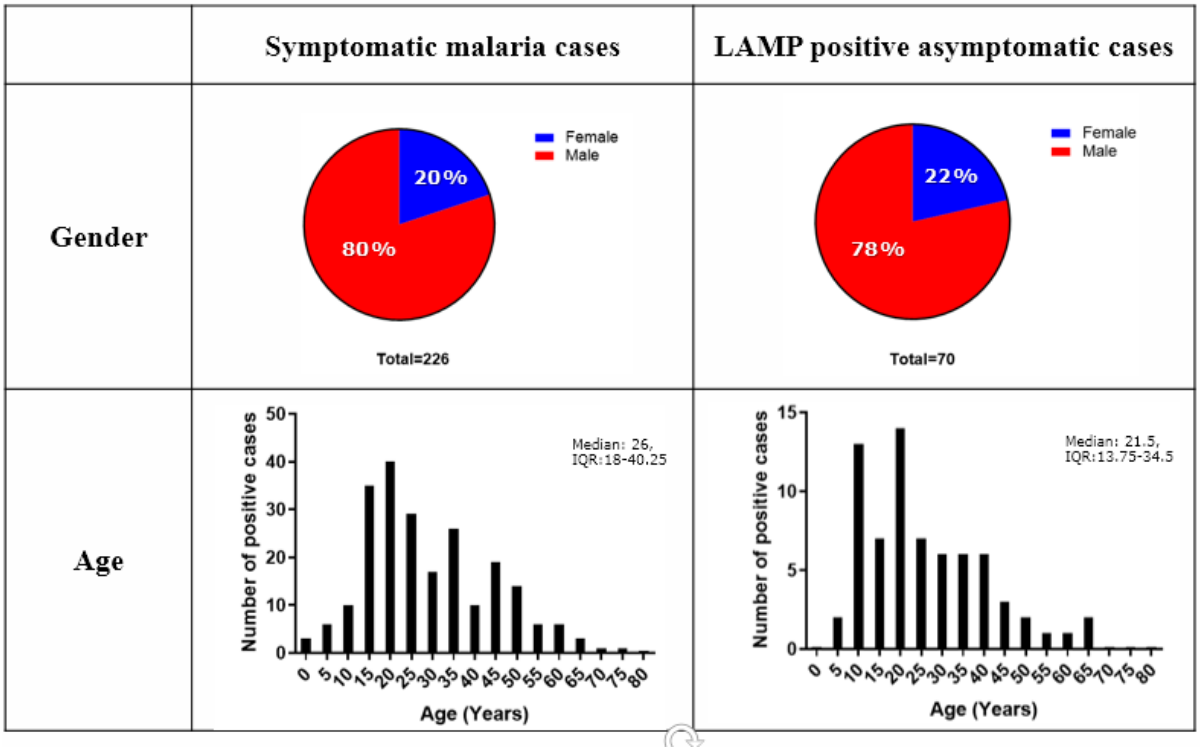

Figure 4.7: The demographics of clinical and asymptomatic cases were comparable.
A higher proportion of males than females experienced both clinical malaria (80% vs. 20%) and asymptomatic infections (78% vs. 22%). Most clinical cases were seen between the ages of 15 and 25 (median of 26 years) while most asymptomatic cases were aged under 20 years old (median of 21.5 years). Thirty-one LAMP positive cases were excluded due to incomplete demographic data sets.

Overlap of LAMP positivity and reported clinical cases

In order to determine any correlation between asymptomatic and symptomatic cases, the LAMP positivity rate of each village was overlayed on top of reported clinical cases in the same area. **Figure 4.8** illustrates this relationship in two communes: Dak O and Bu Gia Map. As GPS information was not collected for LAMP data, positivity rates can only been displayed to the village level resolution (i.e. not down to the household level). Of the participants tested in Dak O, no sampling was conducted in villages 7, 8, 9, and 12. Therefore there was no LAMP data available for these areas. This sampling reflected the logistical limitations of the study. Future studies will need to obtain data and samples from a wider geospatial area to more accurately assess the geospatial transmission. The prevalence of LAMP positivity is indicated by the colour shading intensity, with lighter pinks indicating villages with low LAMP positive rates and darker pink representing villages with high LAMP positive rates. Large red circles highlight villages where symptomatic and asymptomatic cases overlap i.e. indicated by the background heat map in red-orange. There appears to be some correlation between villages with high LAMP positivity and clinical cases, but the villages that were not sampled, makes it challenging to accurately comment on this association. Further testing and analysis is needed. Appendix 6 contains the precise amount of LAMP positive participants by village.

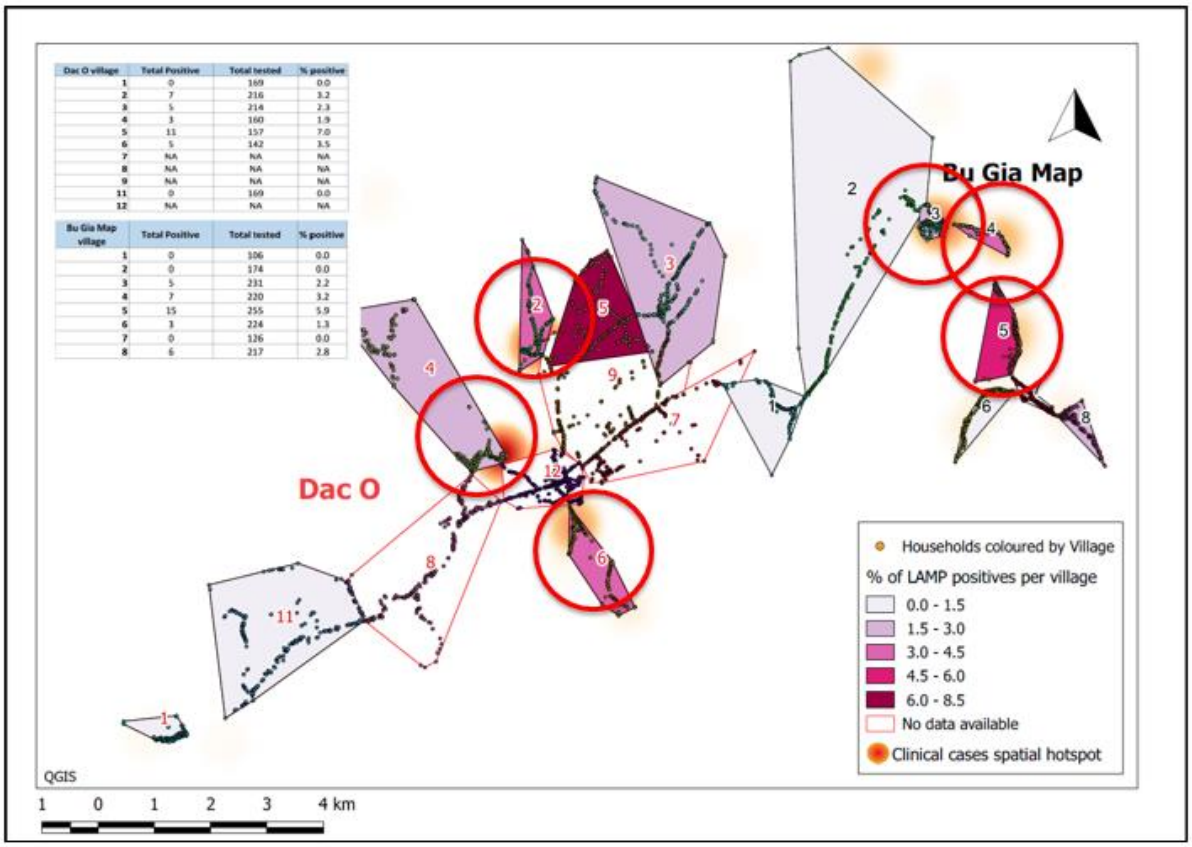

Figure 4.8: Asymptomatic LAMP cases may be able to identify transmission hotpots.

Hotspots in clinical cases were overlapped with LAMP positivity rates. Regions indicating a high prevalence of asymptomatic infection identified by LAMP corresponded with clinically-identified hotspots in villages in Dak O and Bu Gia Map communes (red circles). Polygons are for visualization only and do not correspond to actual village boundaries, clinical cases are illustrated as orange and red hotspots, and the intensity of pink indicates higher proportion of LAMP positive cases found. It should be noted that no data was collected in villages 7, 8, 9, and 12 and thus they have been left uncoloured. StataSE was used to clean data set and QGIS was used to develop map. Map developed by Dr Ricardo Ataide of the Burnet Institute

Potential relationship between interventions and LAMP positivity rates

To investigate whether trends between malaria control interventions and prevalence of asymptomatic infections were apparent, reported interventions were mapped with LAMP positive cases (**Figure 4.9**). As geospatial information was not collected for LAMP data, polygons were used to represent village boundaries for visualization purposes. Of the participants tested, none were from villages 7, 8, 9, and 12 so no LAMP data were available. Households with no interventions were coloured red, IRS households were coloured orange, ITN households were coloured in yellow, and households with both IRS and ITN were coloured in green. Some villages that reported having no interventions (green circle) had a lower LAMP positivity rate than villages that had one intervention in place (blue rectangle). In neighbouring villages with many reported LAMP positive cases, villages that had both interventions in place (red rectangle) appeared to have lower rates LAMP positivity than villages with only one intervention in place (red rectangle compared to blue rectangle).

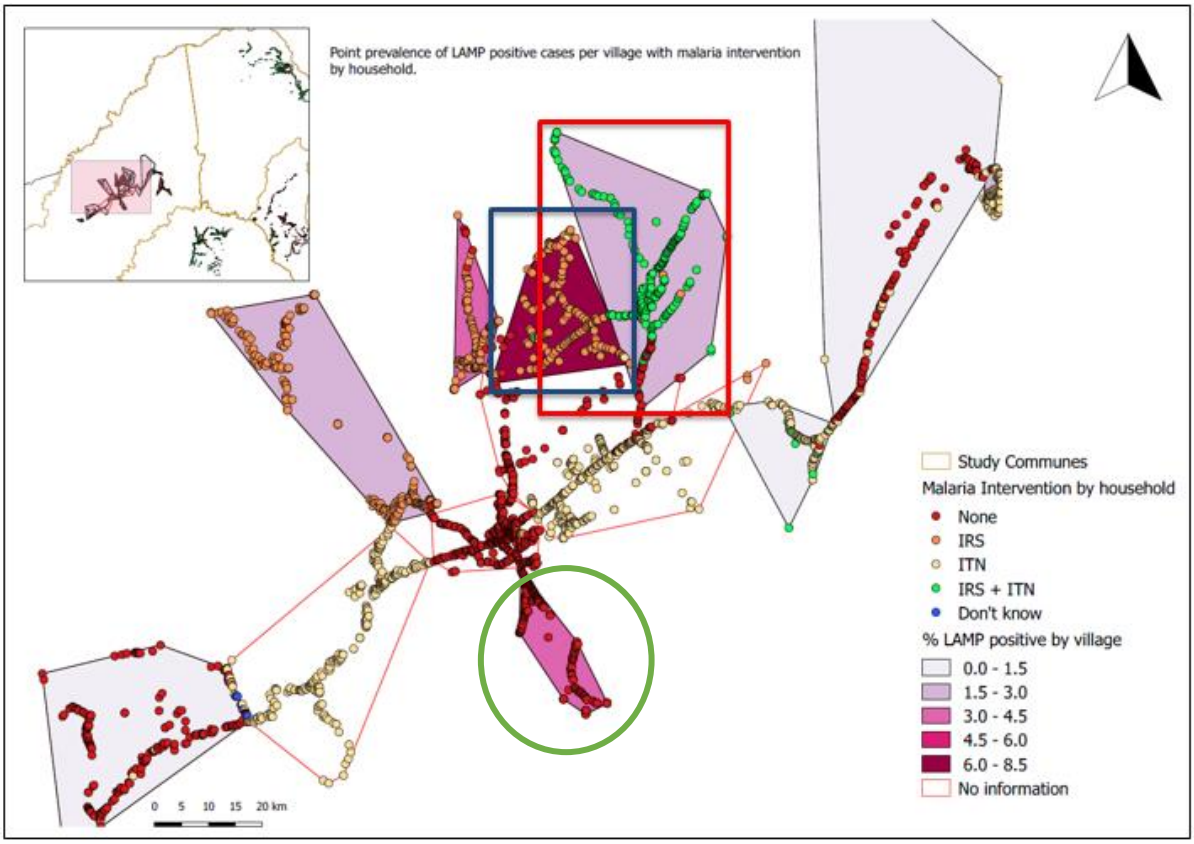

Figure 4.9: Intervention type may impact the prevalence of asymptomatic infections.
Interaction between LAMP cases and intervention was examined. This study was not designed to determine the impact of interventions on cases or asymptomatic infections, however there may be a trend in some villages indicating that more complete intervention packages (ITN and IRS) may have a more significant impact on prevalence of infection (red vs blue squares). Polygons are for visualization only and do not correspond to actual village boundaries and the intensity of pink indicates higher proportion of LAMP positive cases found. StataSE was used to clean data set and QGIS was used to develop map. Map developed by Dr Ricardo Ataide of the Burnet Institute.

Comparison of LAMP and microscopy subset

Figure 4.10 A shows the overlap between LAMP and microscopy detection in the 5,421 participants sampled, with 37 samples detectable by both means. A further 63 samples were detected by LAMP and nine were detected by microscopy and not LAMP. Microscopy data was not reported for one LAMP positive sample and thus was excluded from this analysis. The overall sensitivity and specificity of LAMP compared to microscopy was 80.4% (95% CI, 67-89) and 99% (95% CI, 98-99) respectively (**Table 4.2**). Of the 46 samples reported positive by microscopy, 19 were identified as *P. falciparum*, 10 reported as *P. vivax*, with no data recorded for the remaining 17 positive samples (**Figure 4.10 B**).

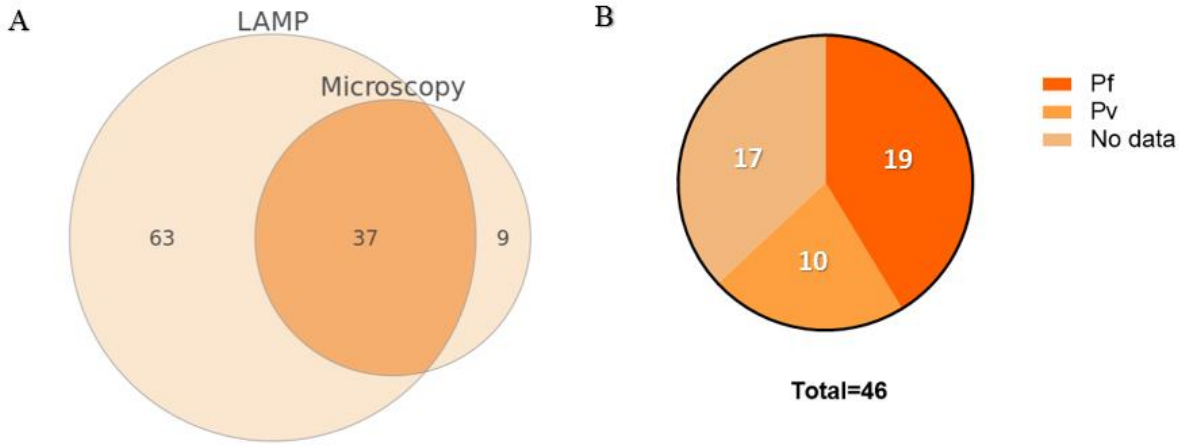

Figure 4.10: Asymptomatic cases detected by LAMP and microscopy.
LAMP and microscopy were compared. **(A)** LAMP and microscopy both identified 37 cases, with LAMP identifying a further 63 that microscopy missed. Nine samples were reportedly detected by microscopy but missed with LAMP. Note: samples that were negative by both LAMP and microscopy were omitted from this analysis **(B)** Of the 46 smear positive samples speciated, 19 were *P. falciparum* and 10 were *P. vivax*. No data were recorded for the remaining 17 smear positive samples so the species of those samples was not known.

Table 4.2: Sensitivity and specificity of LAMP compared to microscopy

		True condition	
		Microscopy positive	**Microscopy negative**
Test condition	**LAMP positive**	37	63
	LAMP negative	9	5311

4.3.3 Validation of LAMP using PCR

Justification of sample set chosen for PCR testing

To validate the in-field LAMP assay, a subset of the samples were tested with qPCR. This subset included all of the samples that were reported as LAMP positive, as well as a proportion of LAMP negative samples. Sample selection was stratified across by commune to ensure representation across the entire geographical region of the study. No LAMP positive samples were available from Dak Nhau but LAMP negative samples from this commune were included. Due to logistical issues of samples transportation (i.e. sample transport from field sites to NIMPE offices) and some instances of having insufficient sample volume to make DBS, only 70 of the 101 LAMP positive samples were accounted for in this subset. LAMP negative samples (in a ratio of three LAMP negative samples to each LAMP positive sample) were randomly selected from each commune to determine the agreement between LAMP and PCR results, including an analysis of sensitivity and specificity. The details of this subset selection and workflow are shown in **Figure 4.11.**

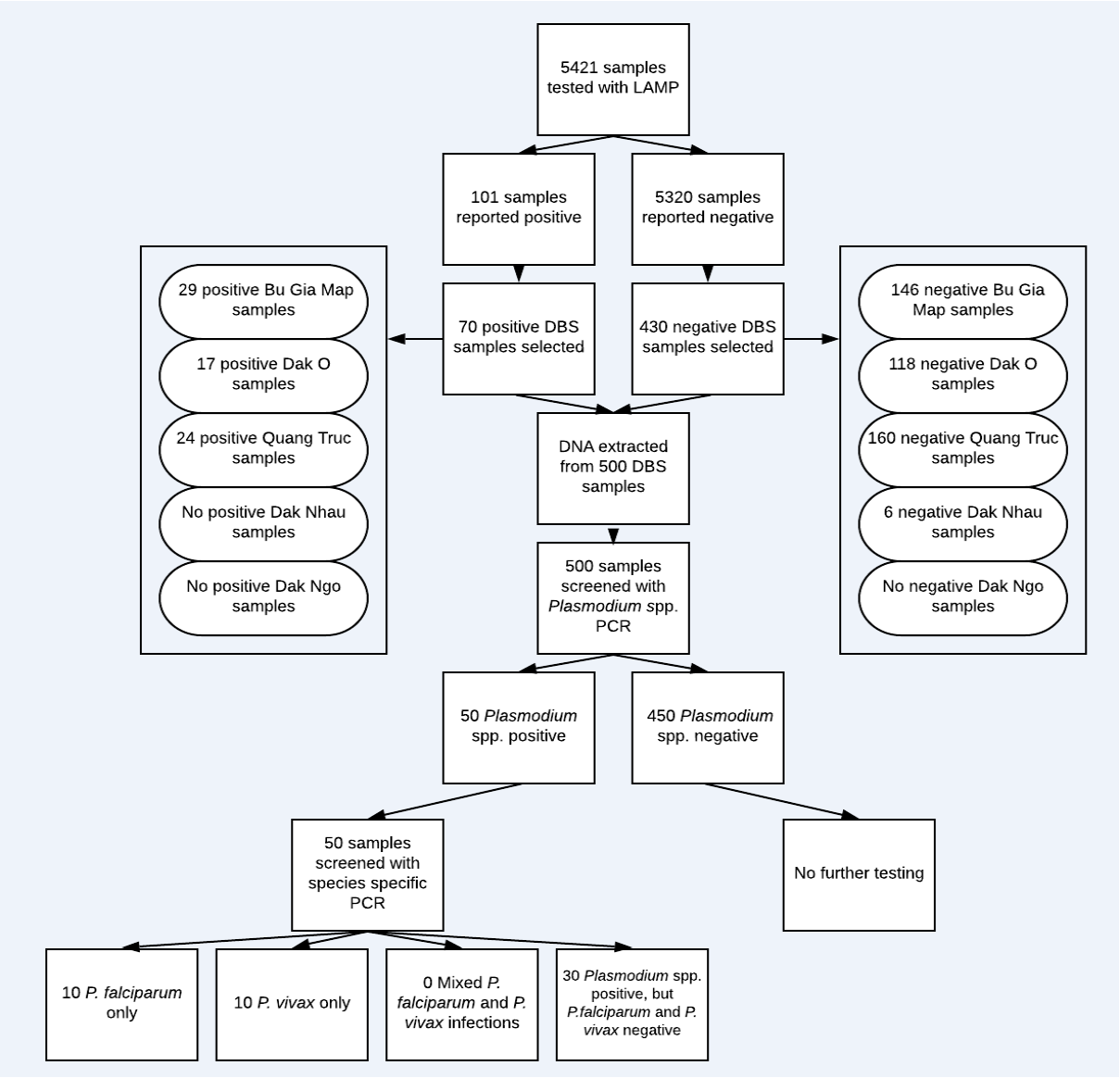

Figure 4.11: Samples selected for PCR testing and speciation.
500 samples from 4 of the 5 Communes were tested with PCR. 50 of these samples were found to be positive when screened with *Plasmodium* spp. assay. These samples were further tested with *P. falciparum* and *P. vivax* specific assays, with 10 different samples found positive for each, and the remaining 30 *Plasmodium* genus positive samples unable to be speciated.

LAMP compared to PCR

Of the 500 samples tested with PCR, 430 were negative. Twenty samples were positive with both PCR and LAMP, 50 were positive for LAMP but negative for PCR, and 30 samples were positive by PCR but negative by LAMP (**Figure 4.12**). The overall sensitivity and specificity of LAMP compared to PCR in this subset was 40% (95% CI, 28-54) and 89% (95% CI, 86-91) respectively (**Table 4.3**). In order to determine whether the samples negative by LAMP but positive by PCR were outside of the LOD of LAMP, the samples were tested with *P. falciparum* and *P. vivax* specific assays which allowed approximate quantification based on a standard curve generated by known amounts of parasite density (**Section 2.5**). Of these 30 samples, it was only possible to quantify two samples (i.e. 28 samples were *Plasmodium* genus positive but negative for *P. falciparum* and *P. vivax* assays). They were both positive for *P. falciparum*, and one sample was estimated to be 14 parasites/µl with the other estimated as 1125 parasites/µl, both within the typical LOD of the Eiken LAMP assay.

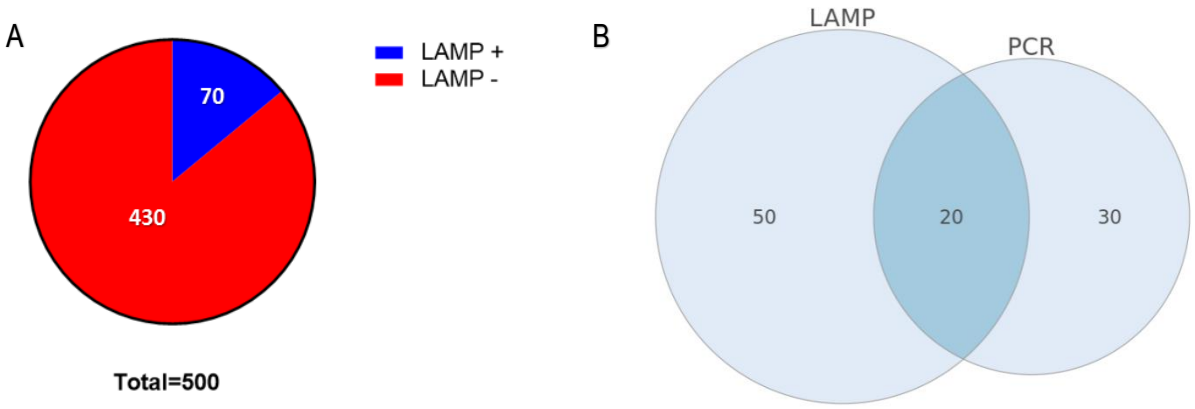

Figure 4.12: Correlation of LAMP and PCR positive samples.
A) 500 samples were tested with *Plasmodium* genus PCR, with 70 being LAMP positives and 430 reported as negative. **B)** Of these samples, 50 samples were found to be positive with *Plasmodium* genus PCR. Twenty of these samples had also been reported positive by LAMP in field, but 30 had not. Fifty of the 70 samples that were originally reported as positive by LAMP were not confirmed positive by PCR. PCR assay was using *Plasmodium* spp 18S rRNA targeted primers. Each samples was tested in duplicate.

Table 4.3: Sensitivity and specificity of LAMP compared to PCR

		True condition	
		PCR positive	**PCR negative**
Test condition	**LAMP positive**	20	50
	LAMP negative	30	400

When comparing the age of LAMP and PCR positive individuals compared to the whole sample population, LAMP positive individuals were similar in age to the sample population; whereas the participants that tested PCR positive were older (**Table 4.4**). When comparing gender, a larger proportion of both LAMP and PCR positive participants were male; whereas the sample population were roughly equally represented by both genders.

Table 4.4: Demographic details of LAMP and PCR positive study participants

Total sampled		
Age	median (range)	24 years (range: 1-97 years)
Gender	female	n=3132 (54.4%)
In-field LAMP positives		
Age	median (range)	23 years (range: 6-68 years)
Gender	male	n=55 (78.6%)
PCR positives		
Age	median (range)	30 years (range: 6-68 years)
Gender	male	n=29 (60.2%)

NOTE: Some participants had incomplete data sets and thus were excluded from this analysis

Speciation and locality of PCR positives

Of the 500 samples tested 50 were PCR positive, but only 20 of these were able to be accurately speciated by PCR. Of these, 10 were *P. falciparum* positive and 10 were *P. vivax* positive (**Figure 4.13**). The remaining 30 samples were either outside the LOD of these assays, or they were positive for another human *Plasmodium* species. The LOD of the *P. falciparum* PCR assay was determined to be 2 parasites/µl based off a standard curve generated by known amounts of *P. falciparum* parasite density (3 separate experiments with each standard in duplicate). The LOD for the *P. vivax* PCR assay was determined to be a hundred-fold less sensitive at 200 parasites/µl. The LOD for *P. vivax* is uncharacteristically high but due to time constraints we were unable to perform further optimisation to rectify this.

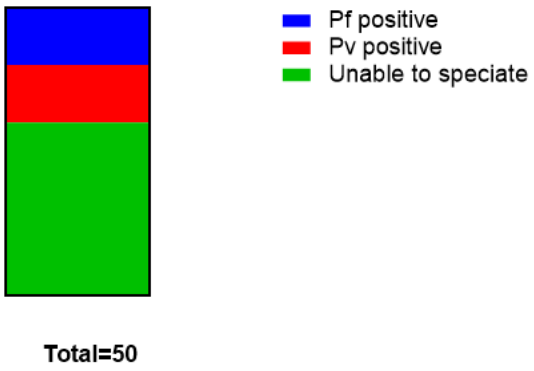

Figure 4.13: Speciation of Plasmodium PCR positive samples.
Of the 50 samples that were *Plasmodium* PCR positive, the species was determined for 20, with 10 samples found positive for each *P. falciparum* and *P. vivax* with no mixed infections. The PCR assay used *P. falciparum* and *P. vivax* specific 18S rRNA targeted primers. Each sample was tested in duplicate.

Of the limited number of samples that could be speciated, there appeared to be differences in species distribution (**Figure 4.14**). Communes such as Bu Gia Map and Dak O contained a mixture of *P. falciparum*, and *P. vivax* positive samples, whereas Quang Truc only contained *P. falciparum* positive samples. The only sample found to be PCR positive from Dak Nhau was unable to be speciated.

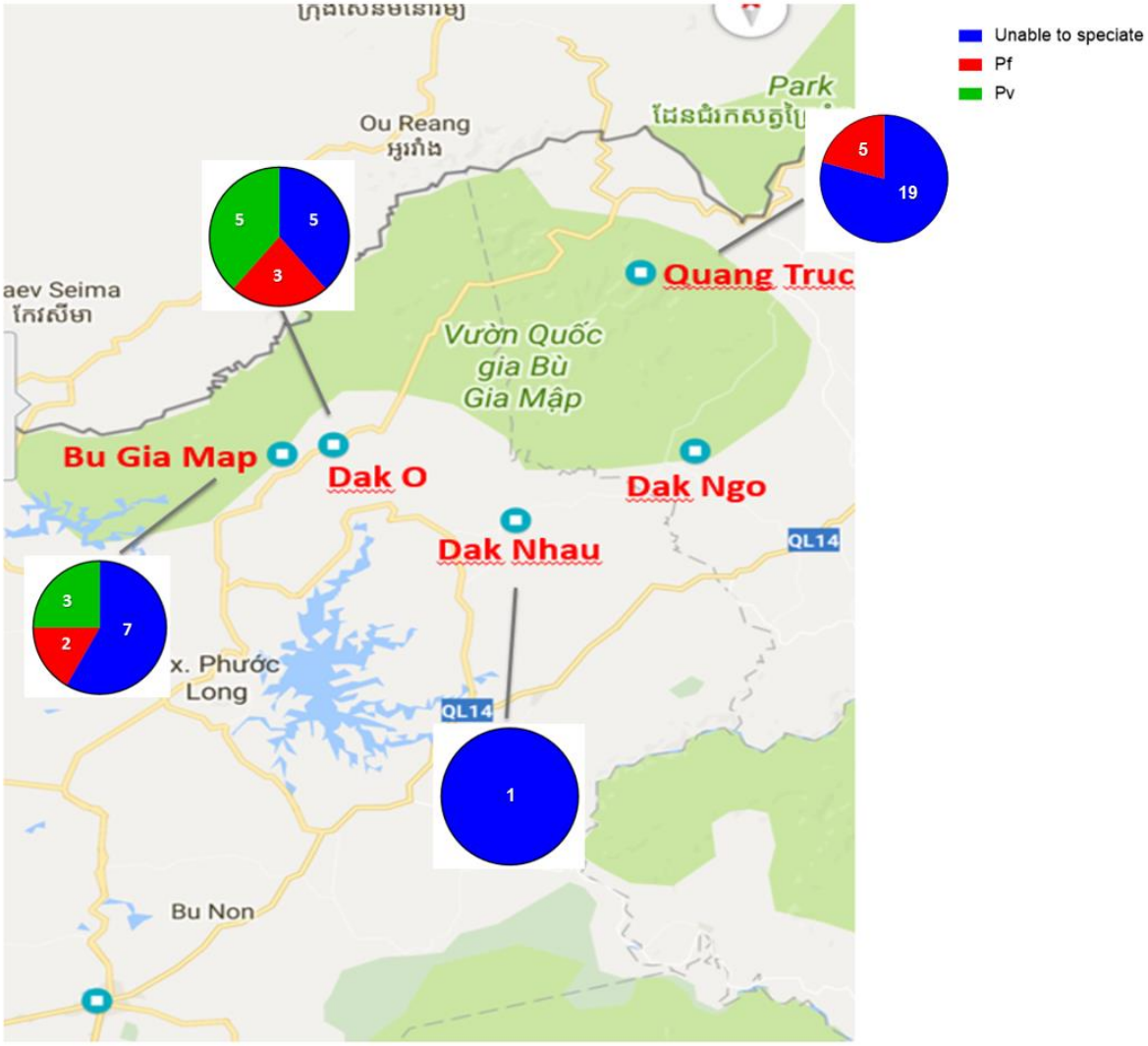

Figure 4.14: Speciation of Plasmodium PCR positive samples according to geographical location. When comparing the species breakdown across different locations, similarities are not seen between neighbouring Communes. It was not possible to speciate the majority of samples in each Commune. Blue circles represent approximate location of each Commune Health Care Centre.

As previously mentioned all *Plasmodium* genus positive samples were subsequently tested with species-specific assays. A standard curve of the species-specific target was run with all samples in these species-specific assays. This parasite density dilution curve ranged from 2,000,000 to 0.02 parasites/µl in ten-fold dilutions. This standard curve was used to determine the parasite density of samples (detailed in **Section 2.5**). All the positive samples were within a range of standards from 80,000 to 0.5 to parasites/µl (**Figure 4.15**). On average, *P. vivax* samples appeared to be of higher parasite density (mean= 6,241 parasites/µl) than *P. falciparum* positive samples (mean=2,263 parasites/µl), but this difference was not significant. Only one sample lay outside the LOD of the standard curve (*P. vivax* positive sample ~40 parasites/µl).

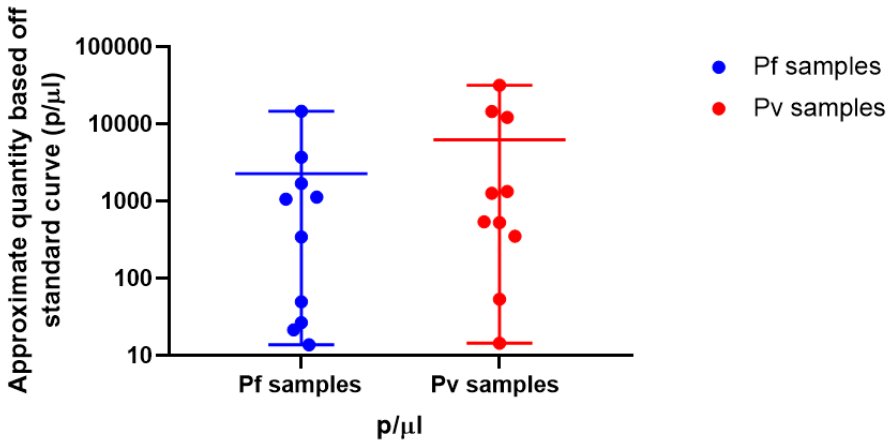

Figure 4.15: Quantification of positive P. falciparum and P. vivax samples.
Samples that were speciated were quantified. Positive samples for both *P. falciparum* and *P. vivax* fell within a standard curve ranging from 0.5 parasite/µl to 80,000 parasites/µl. Standard curve was made with known quantities of the 18S rRNA target gene ranging between $2x10^{6}$ to $2x10^{-2}$ copies. Mean and range are shown. Each sample was tested in duplicate in two experiments.

4.4 Discussion

4.4.1 LAMP was successfully deployed for mass testing on the Vietnam-Cambodia border and was able to identified asymptomatically-infected individuals

Using LAMP to screen 5,421 participants identified an average asymptomatic prevalence of 1.86% in the five communes surveyed. To the best of our knowledge, no other studies have used LAMP at this scale to screen for asymptomatic infections.

In this study, LAMP positivity rates ranged from 0.18% in Dak Nhau to 3.25% in Bu Gia Map. Of the communes sampled, Dak Nhau was the furthest from the forest on the Vietnam-Cambodia border and Bu Gia Map was the closest.

Overall there was a geographical correlation with the hotspots of clinical cases and asymptomatic LAMP positive cases but limitations in the sampling strategy restricted the ability to assess this in detail. Sampling and testing will be required in future studies to ensure that the gaps in village-specific data is overcome. Unlike the clinical cases, LAMP data were only recorded to village level and not to household level, so the same level of detail cannot be shown. Active screening might be pertinent around any clinical cases that present to a Commune Health Care Centres in order to capture the members of the community that are potentially parasite carriers.

4.4.2 LAMP detected more asymptomatic infections than microscopy alone

It has been widely reported that routine diagnostics like microscopy and RDTs have insufficient sensitivity to detect low-density infections that typify asymptomatically infected individuals. For instance, a 2015 study showed that microscopy detected 7.8% of largely asymptomatic individuals compared to 22.6% detected by PCR [16], and a study in Vietnam and bordering countries found that parasite prevalence was 4% by RDT, 5% by microscopy, and 20% by PCR [25]. A substantial amount of the parasite reservoir in Vietnam is being missed by conventional diagnostic tests and remains unreported.

With this in mind, this study demonstrated that microscopy was only able to detect only one third of the of the 101 LAMP positive infections. Because the commercial Eiken LAMP kit is not a quantitative

assay, it was not possible to determine if the infections missed by microscopy were of particularly low parasitaemias below the LOD of microscopy. Similarly, it cannot be confirmed if the infections detected by both microscopy and LAMP were within the LODs of both assays. The observed sensitivity of 80% for LAMP compared to microscopy is lower than previously reported, but the specificity of 99% is representative of the wider literature [103-106] [107-109]. The lower sensitivity was due to nine microscopy positive samples that were reported as being LAMP negative. This result may be due to true assay performance under these field conditions, but may also reflect the challenges that were experienced in data management while undertaking this study: (1) data was summarized by manually checking paper forms and (2) operators may have unintentionally introduced bias when analysing slides. Manually collating these results was laborious and time consuming, and only one staff member was conducting collation. Human error due to fatigue and time pressures could have been possible. Moreover as previously discussed in **Chapter 3**, operators were also observed to re-examine their slides based on LAMP results, despite best efforts to keep these processes blinded. For example, if a sample was determined to be negative by microscopy but then that same sample was found to be LAMP positive, operators would re-examine the slide over more fields and for a longer period of time, actively looking for parasites. To mitigate these confounding factors in the future, the raw data from the paper forms could be re-entered into the database with care; or the original smears could be re-examined, both of which are outside the scope of this thesis however.

4.4.3 The comparison between LAMP and PCR was not definitive when screening asymptomatic individuals in this study

Although PCR was compared to LAMP, a few important differences must be noted: (1) samples were tested with LAMP in the field (Vietnam) by NIMPE operators and samples were tested with PCR in the laboratory (Australia) by a Burnet operator, (2) the sample type used for LAMP was whole blood and the sample type used for PCR was DBS, and (3) the primer target for the LAMP assay was mDNA and the PCR primer target was 18S rRNA.

The discrepancies seen between PCR and LAMP may be attributable to the mistaken reporting of the LAMP result (it was marked as negative instead of positive); or that there are higher copy number of

the 18S rRNA target than the mDNA target in these samples. The sensitivity of LAMP compared to PCR was 40% and specificity was 89%. This sensitivity is uncharacteristically low compared to the wider literature [94, 110-112], and may be the result of the potential limitations mentioned above.

It is difficult to truly compare the two methods and determine which one was more sensitive because of the caveats listed above. Future work will retest these DBS samples in a 18S rRNA LAMP assay to minimise confounding variables. Screening only a subset of samples may have also impacted these results, but testing all samples collected was outside the scope of this thesis. Additional factors that should be considered include the preparation, storage, and transport of DBS samples. When prepared in the field, it was often not possible to keep all samples refrigerated, let alone frozen, due to the volume of samples and the lack of resources at Commune Health Care Centres. Refrigeration was also not possible during transportation from field sites to NIMPE headquarters and once at NIMPE it was only feasible to freeze some samples, while the remaining were refrigerated. DBS were stored under these variable conditions for some months before it was possible to ship them to Australia (again without refrigeration), meaning that samples were tested at earliest six months after they were collected (samples collected at the beginning of the study were stored longer than those collected later in the study). It is not known what effect these time delays and fluctuation in storage conditions may have had on the integrity of the DBS.

As reported, 50 DBS samples were positive with the generic *Plasmodium* genus but only 20 samples were found to be either *P. falciparum* or *P. vivax*. A 2016 WHO report for Vietnam stated the composition of malaria cases were 58% *P. falciparum* and 42% *P. vivax*; though in 2017 it was 64% *P. falciparum* and 35% *P. vivax* (remaining 1% was not accounted for). The NMCP review for Vietnam showed these numbers differed again in 2018, with 55.8% *P. falciparum* and 42.1% *P. vivax* (remaining 2.1% was unaccounted). However, the clinical cases reported during the household mapping survey reported some cases of *P. malariae* so it may be possible that some of the cases identified as *Plasmodium* spp positive are *P. malariae* cases. Several studies have discovered pockets of isolated infections with species other than *P. falciparum* and *P. vivax* as the WHO reports would suggest. A large cross-sectional study of 4,000 participants in Central Vietnam found 210 *P. malariae* cases

(5.25%) [113], a study that surveyed 549 people (both cross-sectional and active case detection) found 32 (5.8%) *P. knowlesi* infections [114], and a more recent survey of 2,303 people in forested areas in Vietnam identified 95 (4.1%) *P. malariae* cases and 19 (0.8%) *P. knowlesi* infections [115]. These and other studies report finding *P. knowlesi* only as co-infections with other species [113, 116, 117], at low densities, and in also in neighbouring countries [113, 114, 117]. Due to time constraints, it was not possible to test samples in this study with *P. ovale*, *P. malariae*, or *P. knowlesi* PCR assays but given the reported cases of these species in the literature, future work should involve testing samples with PCR assays specific for the other human *Plasmodium* species.

4.4.4 Spatial mapping is an important tool in malaria elimination

Using GIS, preliminary relationships between asymptomatic and symptomatic cases, and between interventions and asymptomatic cases were seen. While this study was not designed to measure the interaction between LAMP positivity rates and interventions, some inferences can be made when mapping the two variables against one another. There was a trend in some villages suggesting that a more complete intervention approach, like both IRS and ITN, has more of an impact on the prevalence of infection. However in some villages it appears that the intervention does not impact the number of LAMP positives: some villages that reported having no interventions had a lower LAMP positivity rate than villages that had one or both interventions in place. This may indicate that transmission is occurring elsewhere (i.e. while working in the forest but not in the region around the household itself) and that perhaps interventions should also be in place during periods of work and school, rather than just at the home.

GIS mapping offers many potential applications in moving towards elimination. GIS has been used to monitor vector populations by identifying potential breeding sites and determining the risk posed to humans by those sites (i.e. the proximity to people) [118, 119]. The effect environmental change can have on disease rates has been demonstrated when increases in deforestation [120] and climate change [121] also showed increases in malaria risk. Monitoring the progress of an intervention has also been shown [122, 123]. GIS complementing other tools and data has the potential to contribute greatly to eliminating malaria.

These data also substantiate the significant risk that "cross-border malaria" poses to elimination in the Greater Mekong Subregion. Cross-border malaria stems from control measures being more difficult in areas near country borders because of environmental, political, and socioeconomic factors [124]. Border areas are typically densely forested, are inhabited by *Anopheles* mosquitoes, have little structural shelter to protect inhabitants from mosquitos overnight, and are often inaccessible to health care providers [125]. Political factors can also affect communities living near border areas as countries generally have different malaria control strategies in place. Border countries may have different preventative controls (mainly LLINS or mainly IRS) and different diagnostic modalities (mainly RDT or mainly microscopy) [86, 124]. Commonly, these border areas are inhabited by ethnic minority populations. These populations may have less education opportunities and access to health service. They may also have less exposure to education about use of bed nets, insect repellents and insecticides [41, 126]. These same groups are often mobile and have unofficial status making them even harder to reach by health care providers for diagnostic and treatment purposes [126]. The combination of these factors results in an infected reservoir that can propagate malaria within mobile communities, and also within the fixed populations when migrants return to their respective countries.

4.4.5 Feasibility of LAMP kits as a routine surveillance tool and appropriate screening for elimination settings

From the volume of samples collected during this study, the resource expenditure and logistical demands that came with it, two major findings became apparent. First, that using the Eiken LAMP kits was not affordable for large scale rollout. And secondly, the workflow was also not simple enough to be sustained for long-term mass screening. The low number of LAMP positive results in this low transmission setting suggests that pooling samples before screening with LAMP could be used to dramatically improve cost-effectiveness. The trade-off, however, would potentially be in reduced sensitivity; this issue is explored further in **Chapter 5**.

These results highlight the challenges is mass screening to achieve elimination. The work load and costs of sampling and testing a large number of people was considerable, thus strategies to reduce the number

of people screened (i.e. more targeted focal screen and treat strategies) would be beneficial. Other studies testing the usefulness of molecular methods have used reactive screening methods to ensure the widest coverage of the target population, with some testing a set number of neighbouring households [127] and others testing within a defined radius [128].

The use of a commercially available LAMP assay was selected for use in this project. This was largely due to the procurement processes stipulated in the Global Fund project, and the desire to assess this technology using a well validated LAMP kit. However, the cost of the kits was ~US$14.50 per test (subsided for US$8 per test for the study), thus making the long-term use of these kits unsustainable. This will be further discussed in **Chapter 5**.

4.4.6 Improvement for future studies

Key attributes were identified throughout the course of the study that could be addressed prior to future studies to maximise study outcomes. This included having quality control measures in place for data entry to avoid incorrect, missing, or duplicated entries, mitigating aspects where operator bias could occur and ensure they do not impact data outputs, and ensuring that the same sample type and equivalent parameters could be used when comparing assay types such as when comparing LAMP and PCR.

4.5 Conclusion

This study provided valuable insights into the asymptomatic reservoir that exists in Vietnam. LAMP was found to be a sufficiently sensitive tool to detect asymptomatic infections, but in the current format (Eiken kit) is not practical or cost effective for large scale use in resource constrained countries. Further testing must be carried out in order to definitively assess the effectiveness of LAMP over PCR and microscopy, and targeted screening may uncover more underlying infections. Once detected, these cases must then be appropriately managed or treated in order to progress towards elimination.

5 Development of an in-house LAMP assay for the detection of low-level *Plasmodium* parasitaemia

5.1 Introduction

Malaria diagnostic assays in widespread use lack the sensitivity to detect low parasite burdens in people with asymptomatic infections [16, 24]. This current lack of a sufficiently sensitive and rapid diagnostic test facilitates an ongoing, undetected, reservoir of *Plasmodium*. To effectively address the problem of asymptomatic carriers acting as a reservoir of infection, one public health strategy is to implement MSAT of those that are positive (compared to mass drug administration which occurs without testing). In MSAT all individuals in at-risk communities would require screening, ideally using a highly sensitive diagnostic test [129, 130]. A test with a short turnaround time would facilitate immediate treatment of positive individuals. A MSAT approach requires screening hundreds, if not thousands, of people in any one area; thus requiring ease of sampling, low cost, easy to use tests and with rapid generation of results.

LAMP technology has the potential to change the landscape of infectious disease diagnostics, as it allows for the integration of genetic approaches with POC testing. The availability of such tests in low-income, resource-poor settings has the potential to achieve the increased sensitivity and specificity required to support the elimination of malaria [96, 127, 131]. A commercial LAMP assay is available for the diagnosis of *Plasmodium* genus, *P. falciparum*, and *P. vivax*. The kits are reported to be capable of detecting infections as low as 1 parasite/µl with a sensitivity of 96-100% and specificity of 97-100%; although the manufacturer does not state what diagnostic test is being evaluated against [2]. However, at the time of writing, each test cost ~US$14.50. Current malaria RDTs typically cost ~US$1.00 when subsidised through mass procurement mechanisms in resource-limited settings [132, 133]. Although RDTs generally lack the sensitivity required for detection of asymptomatic malaria infection, they set the benchmark of the price of a malaria diagnostic test. As such, to be broadly applied, a LAMP diagnostic test would need to have a similar cost.

In addition to financial constraints, health care facilities in low-middle income countries are commonly stretched for resources and time. New diagnostic tests need to be easy to use and rapid. Existing commercial LAMP kits, while effective, require reliable electricity and trained personnel. In areas of low transmission, the populations at most risk and the location of health care centres often do not

overlap. Therefore, it is vital that deployed technologies are applicable for use in remote settings, where malaria elimination is likely to be hardest to achieve [134].

The ASSURED (**A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment- free, and **D**eliverable to end-users) criteria can be used as a standard in identifying features of diagnostic tests that may be successfully deployed and sustained, especially in malaria elimination settings [135]. Existing commercial LAMP kits show great promise as they are sensitive and specific, but they are not affordable in the context of mass screening. They also do not have a simple enough workflow and are not as rapid as RDTs and microscopy. Consequently, this study seeks to develop a cost-effective LAMP assay that meets most of the ASSURED criteria. This work assesses the sensitivity and specificity of the assay; and compares the developed in-house assay to a commercially available LAMP assay and to PCR, the latter being the current gold standard in malaria diagnosis for high-sensitivity testing. The chapter explores novel methods of parasite detection and discusses the potential of the assay as a field-deployable diagnostic tool.

5.2 Methods

5.2.1 Comparison of dyes for detection of positive results using in-house LAMP assay

Protocols for each detection method are described in full in **Section 2.4**. In brief, SYBR Green and calcein were assessed as possible detection modalities. When SYBR Green was used for detection, 1 µl of a 1:4 dilution (10,000x SYBR stock with dH_20) was added post-amplification; and when calcein was used, 2.5 µl of a 5 mM calcein and 10 mM $MnCl_2$ mix was added pre-amplification (after a serial titration was used to determine the optimal concentrations of each). For both dyes a positive reaction was observed as green under white light and fluorescent under UV light. For calcein detection determined by isothermal instrumentation (T16-ISO, Axxin Pty Ltd), detection in the FAM channel (495 nm excitation, 515 nm emission) was used with an LED intensity of 18%. Three LAMP runs were performed for each condition with each sample in duplicate.

5.2.2 Optimization of in-house LAMP assay

LAMP mastermix optimization

The detailed protocols for the in-house LAMP assay are described in **Section 2.4**. In summary, the optimized LAMP assay consisted of 2.5 µl 10× isothermal buffer, 5 µl of primer pool, 1.5 µl 100mM $MgSO_4$, 3.5 µl 10mM dNTP mix, 1 µl 8000U/µl *Bst* WarmStart 2.0 polymerase, 7 µl NFW, and 2.5 µl of a mix which was equal parts 5 mM calcein and 10 mM $MnCl_2$. An additional 2 µl of template (either sample or control) was added to make up to a final volume of 25 µl. When using the T16 instrument, volumes of all constituents were doubled to create a reaction volume of 50 µl. Incubation temperature and duration were also optimised. Three LAMP runs were performed for each condition with each sample in duplicate.

Primer target optimization

Two primer targets were compared to determine which was more sensitive. One set targeted the 18S rRNA [70] subunit and the other targeted the mDNA [71] of the *P. falciparum* genome. Previously published primers were synthesised by Geneworks Pty Ltd. Lyophilized primers were reconstituted to 100 µM using NFW and working primer pools of 1.6 µM FIP and BIP, 0.2 µM F3 and B3, and 0.4 µM Loop F and Loop B were made. A 5 µl aliquot of this primer pool was added to each LAMP reaction. Three LAMP runs were performed with each primer target and the primer set which achieved greater sensitivity was used for all subsequent LAMP reactions.

5.2.3 In-house LAMP compared to PCR

The optimized in-house LAMP reaction (**Section 5.2.2**) were compared to PCR (**Section 2.1.3**) using DNA extracted from parasite density titrations (**Section 2.3.1**).

5.2.4 Cost analysis

A simple cost analysis was performed to compare the in-house LAMP assay to the Eiken LAMP assay. The costs used in this analysis were based on the actual costs of the RAI study described in **Chapter 4**. Costs were assessed for a once-off study, and also for a hypothetical period of five years where a comparable number of participants (to that of the RAI study) were screened each year. For the second phase of analysis, equipment costs were not carried over (i.e. assuming there were no maintenance requirements or failure in equipment) only the costs directly associated with LAMP testing. Labour costs were not included in this analysis as they are highly variable depending on the health system and funding structure used to implement MSAT with this type of technology.

5.3 Results

5.3.1 Comparison of dyes for detection of positive results using in-house LAMP assay

Before undertaking optimisation of each of the LAMP assay components, a suitable detection method was determined. SYBR Green is commonly used to detect DNA amplification in PCR assays and was initially used in the development of this in-house assay. SYBR Green added before the amplification was inhibitory to the LAMP reaction, as shown by the lack of a colour change, UV fluorescence (**Figure 5.1 A; tube 1**) and absence of band in an agarose gel (**Figure 5.1 B; lane 1**). Addition of SYBR Green after the LAMP amplification produced a strong colour change in positive samples, i.e. from orange to green under visual white light, fluoresced brightly under UV light (**Figure 5.1 A; tube 3**) and produced the typical laddering effect seen with multiple amplicon sizes seen in LAMP (**Figure 5.1 B; lane 3**). The addition of SYBR Green after amplification posed a potential hazard for amplicon escape and cross-contamination of samples, so a detection modality that could be added pre-amplification was assessed.

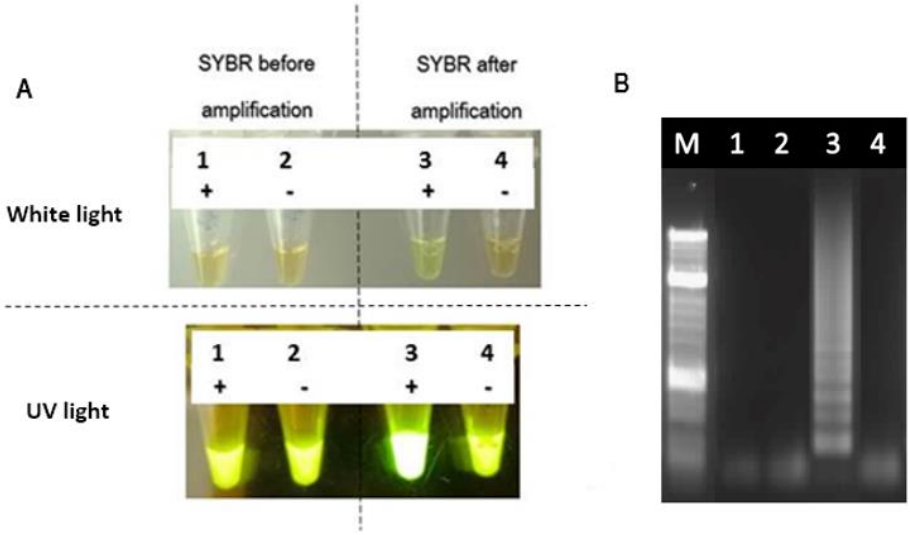

Figure 5.1: LAMP reaction detected with SYBR Green.

In-house LAMP assay with SYBR Green added before and after amplification. **(A)** Absence of colour or fluorescence change in the positive sample (tube 1) and **(B)** absence of products on 1% agarose gel (lane 1) indicated inhibition. Samples from left to right are positive control with SYBR Green added prior to amplification, negative control with SYBR Green added prior to amplification, positive control with SYBR Green added post amplification, and negative control with SYBR Green added post amplification. Positive controls were purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage. NFW was used for negative controls.

Calcein (quenched by the presence of Mn^{2+} ions) has been shown in the literature as a detection modality for LAMP and relies on the loss of quenching that occurs when manganese binds to the pyrophosphate ions produced during DNA synthesis (a visible precipitate of manganese pyrophosphate is also produced). Calcein and $MnCl_2$ were titrated against each other to determine the most distinct colour change between positive and negative. Clearest distinction between positive and negative results was achieved when calcein (5 mM) and $MnCl_2$ (10 mM) were combined in equal parts and 2.5 µl of this mixture was added to LAMP reactions, giving final concentrations of 250 µM calcein and 500 µM $MnCl_2$ in a 25 µl reaction **Figure 5.2**.

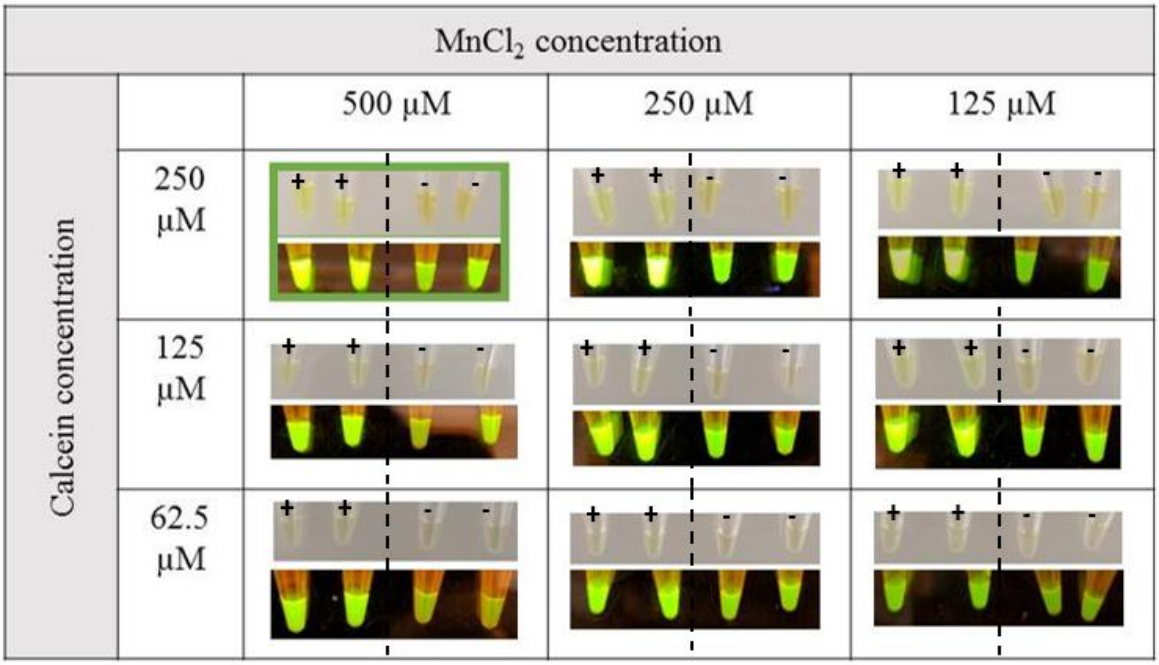

Figure 5.2: Visual differentiation between positive and negative LAMP results using calcein.
Calcein and $MnCl_2$ were titrated to determine optimal concentrations. The clearest distinction under natural and UV light was observed with calcein at 250 µM and $MnCl_2$ at 500 µM (upper left). These concentrations were used in subsequent reactions. Positive and negative controls were tested in duplicate for each permutation of calcein and $MnCl_2$. Positive controls were purified genomic DNA extracted from cultured 3D7 laboratory strain ***P. falciparum*** parasites at a parasitaemia of 4% ring stage. NFW was used for negative controls.

5.3.2 In-house LAMP assay optimization

Use of the T16 isothermal amplification instrument compared to visual readouts

Visual end-point reads of LAMP reactions have clear advantages for simple and affordable utility. However there are limitations, especially when quantitative results are required, as is the case for assay optimisation. To afford greater insights into assay development, the commercially available T16-ISO (**Figure 5.3 C**) isothermal amplification instrument (Axxin Pty Ltd) was used for further optimization of the in-house LAMP assay. The T16 was used to record amplification in real time allowing for temporal assay optimisation which was more precise than single time-point visual optimisation. No difference can be seen between different parasite positive DNA samples (500 parasites/µl, and 50 parasites/µl) when observed visually; however there was a clear dose titration effect seen using the T16 real-time read out (**Figure 5.3**).

Using this instrument, each component of the LAMP assay was either optimized individually or in conjunction with a related component.

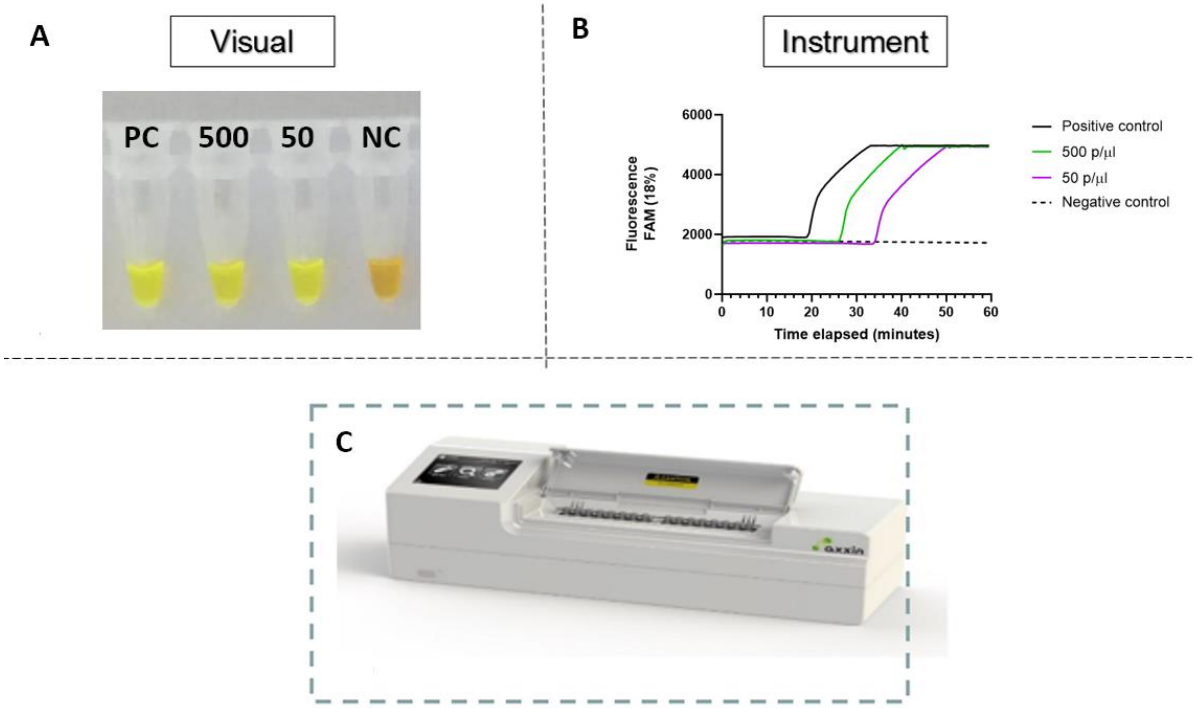

Figure 5.3: T16 detection provided more valuable information compared to visual detection alone

Instrument detection was compared to visual detection. **(A)** Limitations of visual detection alone wherein only a colour difference between positives and negatives can be seen (left to right: positive control, 500 parasites/µl, 50 parasites/µl, and negative control), whereas **(B)** shows the much more informative output of the T16 **(C)** enabling the distinction between different positive samples. Positive samples were purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites spiked into whole blood at concentrations of 500 and 50 parasites/µl. NFW was used for negative control.

Overall sensitivity was not impacted by primer target

Two primer sets were compared to determine which were more sensitive. Sensitivity did not differ between the two primer targets (both able to detect $1x10^{-3}$ ng/µl of genomic DNA, represented as the 1:10,000 dilution); however, the time to amplification was faster with mDNA primers than the 18S rRNA primers (**Figure 5.4**). As a faster time to result is desirable for field applications, mDNA primer targets were used for all subsequent LAMP reactions.

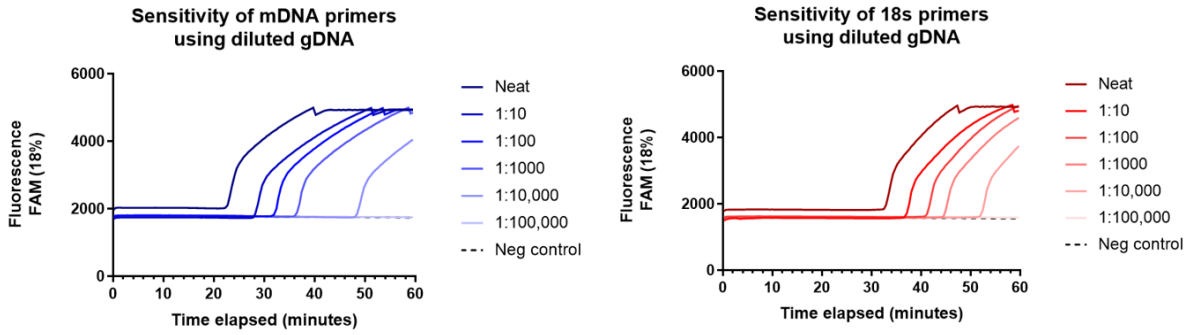

Figure 5.4: Effect of primers on amplification.
(A) mDNA and **(B)**18S rRNA targeted primers were compared to determine which primer set would be more sensitive. Primers were tested with a titration of kit extracted genomic DNA from cultured parasites. The undiluted DNA was at a concentration of 10 ng/µl. Both primer targets achieved the same sensitivity with an LOD of $1x10^{-3}$ ng/µl (1:10,000) but there was a faster time to result with the mDNA primers. Positive samples were purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites. NFW was used for negative control.

Optimal incubation temperature occurs at 65°C for mDNA primers

The optimal incubation temperature for the in-house LAMP assay was determined using the T16. mDNA primers were tested in the LAMP assay over different temperatures to determine which temperature was most suitable for these primer targets. The assay was tested over a 60 minute incubation at 50 °C, 55 °C, 60 °C, and 65 °C with all other reagents remaining unchanged. The most efficient amplification defined by the shortest time of amplification initiation occurred at 60 °C. However, negative samples also appeared to amplify at that temperature, so 65 °C was chosen for subsequent assays (**Figure 5.5**).

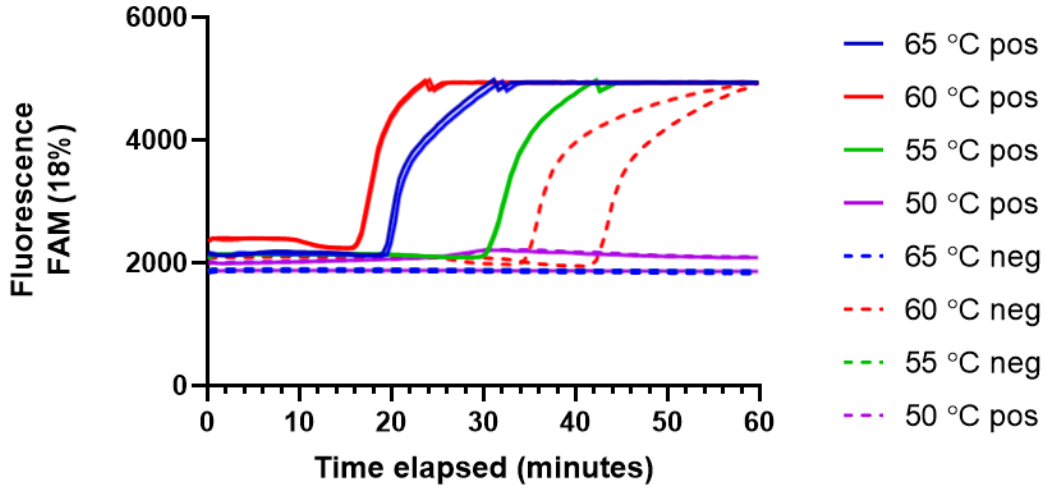

Figure 5.5: Effect of incubation temperature on amplification.
The effect of incubation temperature was assessed. Amplification occurred at 55 °C, but at a slower rate than at 60 °C and 65 °C (no amplification occurred at 50 °C). Although time to amplification was faster at 60 °C false positives also occur at this temperature, therefore 65 °C was deemed to be optimal. mDNA target primers were used in all samples. Positive controls were purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage. NFW was used for negative controls.

dNTP concentration influences occurrence of false positives

Optimisation of dNTP concentration was conducted by testing concentrations ranging between 0.4 mM and 1.9 mM. dNTPs at a concentration of 1.9 mM produced the strongest amplification in a positive sample, but resulted in late (i.e. 55 minutes), non-specific amplification in the corresponding negative control (**Figure 5.6**). Therefore, a dNTP concentration of 1.4 mM of dNTPs was used for subsequent experiments.

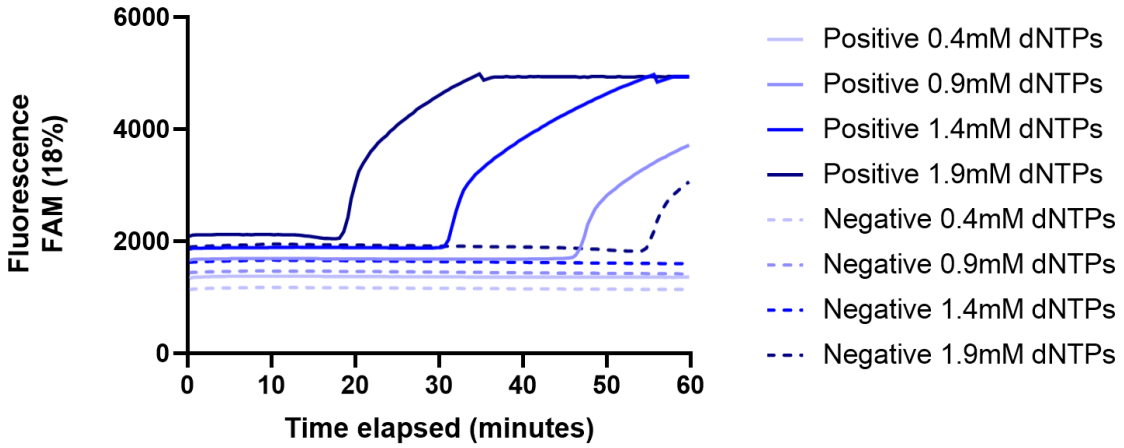

Figure 5.6: Effect of dNTP concentration on amplification.
LAMP assays were tested with increasing concentrations of dNTPs while all other reagents concentrations remained unchanged. In positive samples, no amplification occurs at 0.4 mM dNTPs, weak amplification occurs at 0.9 mM dNTPs, and strong amplification at 1.4 mM and 1.9 mM dNTP. Although strong amplification occurs at 1.9 mM dNTP, false positives can also occur in negative samples, therefore 1.4 mM dNTPs were used in subsequent assays. mDNA target primers were used in all samples. Positive samples were purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage. NFW was used for negative controls.

In-house LAMP assay can be shortened to 45 minutes

To determine time to positive result, serial dilutions of kit extracted DNA were used in LAMP incubated for 60 minutes. After 30 minutes, the positive control and samples ranging between 500 parasites/µl and 50,000 parasites/µl amplified. At 50 parasites/µl amplification had just initiated, so fluorescence was low. After 45 minutes, 50 parasites/µl and 5 parasites/µl samples amplified. Continuing incubation to 60 minutes did not increase sensitivity or produce false positives (**Figure 5.7**). A 45 minutes LAMP incubation was sufficient to detect positive samples in this experiment, thus was used as the temporal cut-off for subsequent LAMP assays.

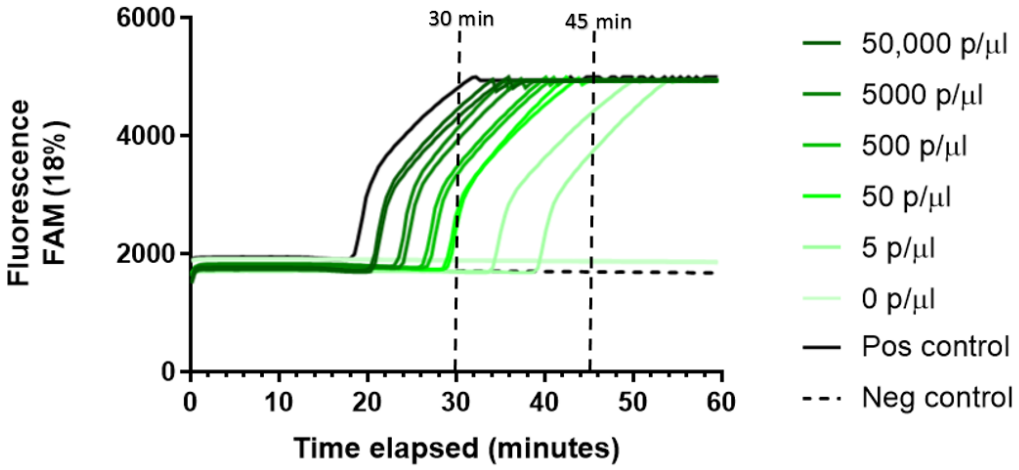

Figure 5.7: Effect of assay duration on amplification.
Minimum duration of incubation was evaluated. While 30 minutes was sufficient for the complete amplification of strong positives (50,000-500 parasites/µl), it may miss samples lower than 500 parasites/µl. A 45 minute incubation was sufficient for moderate to low positives ranging between 50 and 5 parasites/µl, and incubating for 60 minutes did not impact limit of detection. mDNA target primers were used in all samples. Positive controls were purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage. NFW was used for negative controls.

5.3.3 In-house LAMP assay compared to qPCR assays

The optimized in-house LAMP assay was compared to an optimised in-house qPCR assay. The PCR assay uses a 18S rRNA primer target and is not compatible with boil and spin extracts (unlike the Eiken LAMP kit) so commercial kit extracted DNA were used to test both the PCR assay and the in-house LAMP assay. A summary of each assay is provided in **Table 5.1**. The sensitivity of each assay was determined using a titration of parasites ranging from 50,000 – 0.5 parasites/µl (**Figure 5.8**). Each titration was repeated in duplicate in two separate runs. The LOD of the PCR assay was found to be 0.5 parasites/µl, while the LOD of the in-house LAMP assay was found to be 50 parasites/µl. This resulted in a sensitivity of 67% (95% CI 39-86) and a specificity of 100% (95% CI 17-100) of LAMP compared to PCR.

Table 5.1: Summary of molecular assays used to validate in-house LAMP reaction

	In-house LAMP	**qPCR**
Primer target	mDNA	18S rRNA
Extraction method	Commercial kit	Commercial kit
Amount of sample added	4 µl	2.5 µl
Total reaction volume	50 µl	10 µl
Incubation parameters	65 °C for 60 minutes	94 °C for 2 minutes 40 cycles of 94 °C for 15 seconds 60 °C for 1 minute

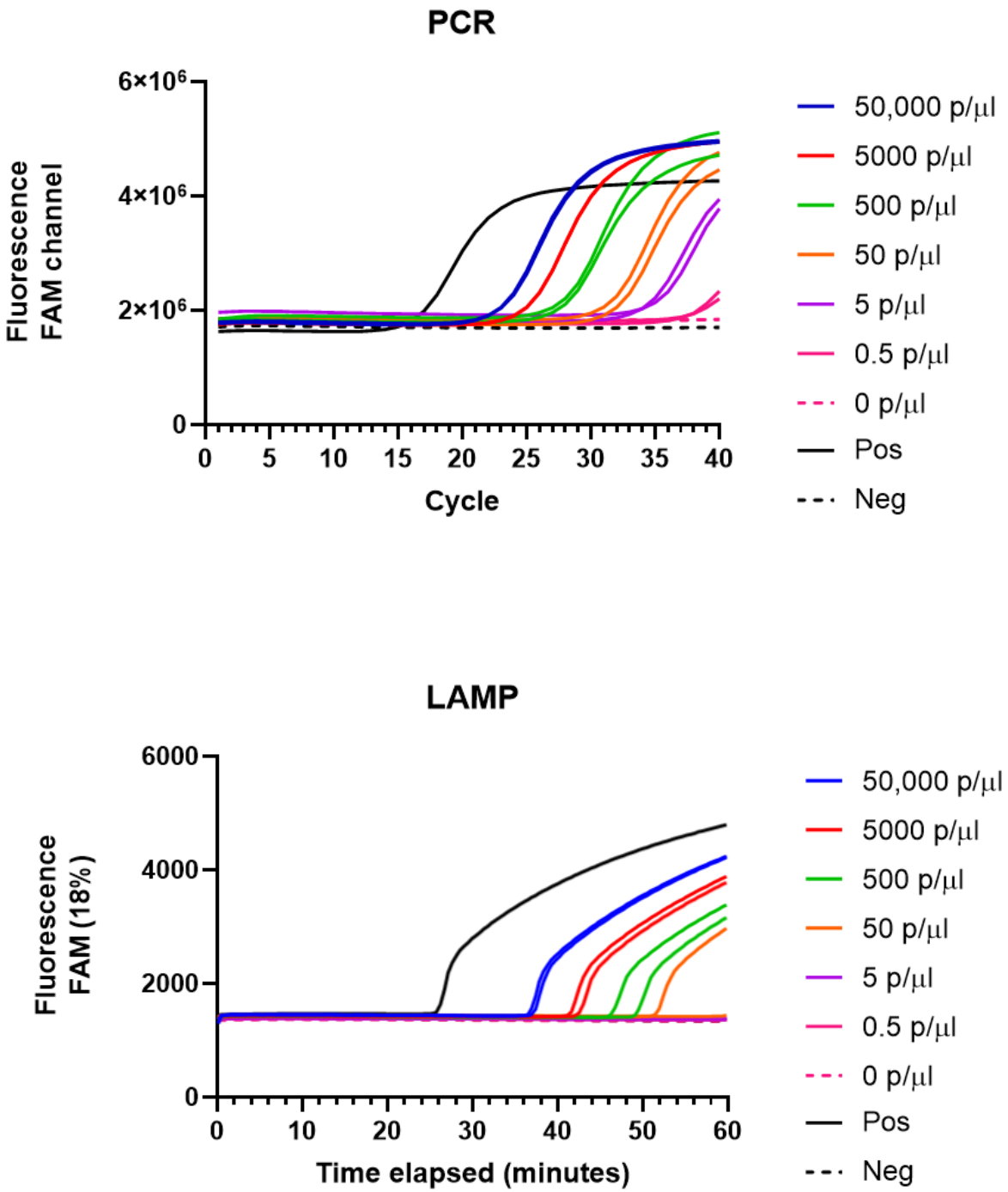

Figure 5.8: In-house LAMP assay compared to PCR

The in-house assay was compared to PCR. Using a parasite titration of 50,000-0.5 parasites/µl the LOD for the PCR assay was 0.5 parasites/µl **(A)** and the LOD for the in-house LAMP assay was 50 parasites/µl **(B)**. *P. falciparum* 3D7 laboratory strain parasites were used for the titration and the positive control (4% parasitaemia) wherein whole blood was spiked with parasites and then extracted with a commercial kit. NFW was used for negative control. This figure show duplicates of each sample, and is representative of two separate experiments with each sample performed in duplicate.

5.3.4 Cost analysis of in-house LAMP compared to Eiken LAMP kit

Table 5.2 shows a brief breakdown of the costs included in the analysis. Using the in-house assay LAMP assay to test 5000 participants in a study would cost US$19,616.60 compared to US$79,961.00 when using the Eiken LAMP kits; a saving of US$60,345.00. If the same number of people were routinely screened every year over 5 years, a total saving of US$260,245.00 would be achieved using the in-house LAMP assay (**Appendix 4**). Cost savings would be even greater if implemented across a national program. A more detailed cost analysis can be found in **Appendix 4**.

Table 5.2: Breakdown of expenditure included in cost benefit analysis

	Cost (US$ associated with 3 month study of 5000 participants)	
Resource	**Eiken LAMP assay**	**In-house LAMP assay**
Equipment		
Pipettes (set of 4)	1,435.50	1,435.50
Microcentrifuge (x1)	2,444.20	2,444.20
Heat block (x1)	845.00	N/A
Heat block insert (x1)	400.00	N/A
T16 (x1)	N/A	9,000.00
Reagents		
Eiken LAMP kits	72,500.00	N/A
In-house LAMP assay	N/A	4,400.00
Consumables		
Pipette tips	628.10	628.10
Eppendorf tubes	548.80	548.80
Gloves	1,160.00	1,160.00
LAMP tubes	N/A	623.70
TOTAL	79,961.60	19,616.60

5.4 Discussion

5.4.1 Cost effective molecular screening tools are needed in elimination settings

In endemic countries that are currently on track to eliminate malaria, very little of the available funding is allocated to diagnostic testing [1]. This limited funding would need to be utilized effectively and the Eiken LAMP kit that costs upwards of US$14.50 (assuming a primary screen with the *Plasmodium* genus test and then confirmatory species specific tests) would not be appropriate due to cost. Directly comparing the sensitivity of the in-house LAMP assay to the Eiken LAMP kit was outside the scope of this thesis, however a simple cost analysis showed substantial savings when using the in-house LAMP assay. The analysis could be expanded to include ongoing equipment maintenance and labour costs, but this was outside the scope of this analysis. Despite the simple nature of this cost analysis, it demonstrates the considerable different in cost of the Eiken assay and this in-house assay. The in-house assay is more affordable if used in a single tear study and if it were used over consecutive years in ongoing surveillance.

The in-house LAMP assay developed in this study was optimized for visual detection, so that equipment would not be essential in resource poor settings. The visual assay was then made compatible with instrumentation so that it could also be used in established hospital settings to gain information beyond the presence or absence of infection (i.e. could distinguish between different parasite densities). Instrumentation also allowed for a more thorough optimization of the in-house assay including optimisation of reagent concentration, incubation temperature, incubation duration, and primer target. With these optimized conditions the in-house assay had a consistent limit of detection of 5 parasites/µl.

A 2018 study by Zelman *et al.* compared the costs associated with LAMP (Eiken kits) and microscopy in the context of reactive case detection. Microscopy specific costs equated to US$0.62 per individual screened and LAMP related costs were US$16 per individual screened (and US$9 per individual in subsequent years). Personnel costs accounted for the majority of microscopy related costs and consumables accounted for the majority of LAMP related costs [136]. This study stated that LAMP costs could be greatly reduced if kits were subsidized or a less expensive variation was used.

LAMP also has cost benefits outside of mass screening in pre-elimination malaria endemic settings. Cheaveau *et al.* (2018) trialled LAMP in a hospital setting in Canada testing returned travellers with malaria symptoms. CDC guidelines recommend RDTs as a preliminary test, followed by thick and thin smears every 6-8 hours on average three times to ensure that no parasites are present. This methodology is time and cost exhaustive in a non-endemic hospital setting, and LAMP was found to equate to a cost saving of up to US$13 per person [137].

There is considerable scope for using LAMP in the diagnosis of other infectious diseases, and its application could save money. A cost analysis comparing LAMP and PCR for TB detection found LAMP cheaper to perform and implement. In peripheral laboratories in Malawi and Vietnam, the average cost per test of LAMP was between US$13.78- US$16.22 compared to US$19.17- US$28.35 for PCR [138]. LAMP was also cheaper to implement accounting for 9.33% and 17.2% of the allocated TB control budgets in Vietnam and Malawi respectively compared to 18% and 37% for the PCR assay. In rural settings LAMP is generally a more affordable molecular test than PCR [138].

5.4.2 Comparisons with other NAATs

The sensitivity of the in-house LAMP assay was not comparable to the sensitivity of PCR (the gold standard of NAATs). A decline in the performance of in-house assay was apparent when this testing was conducted, as was illustrated by the slower time to amplification and the decrease of fluorescent signal (**Figure 5.7** compared to **Figure 5.8**). The reasons behind this are unknown. Measures were taken to attempt to rectify this problem such as synthesising new primers, purchasing new lots of all reagents, and re-calibrating the T16 instruments; all to no avail. Optimising the assay with another detection dye, including additives such as betaine or BSA to improve polymerase accessibility, or designing new primers, may have also improved detection. However due to time constraints these methods were not pursued. Future work will further investigate factors that may contribute to the decline in performance. That could lead then to a re-evaluation the comparison between LAMP and PCR once the in-house assay returns to acceptable levels of sensitivity.

5.4.3 Comparisons with other in-house assays

Many research groups have recognized the gap currently faced by malaria diagnostics, and as such have developed their own in-house assays. Most use the 18S ribosomal subunit as a primer target [70, 110, 139, 140], few use mDNA targets [71, 141], and even fewer target unique regions such as alpha tublin (Dinzouna-Boutamba, 2014) and subtelomeric sequences [54]. Detection methods also vary between assays with some opting to use calcein as a detection method [61, 143], others using hydroxynapthol blue (another metal ion indicator) [141, 144], DNA binding dyes such as SYBR [145] gel electrophoresis or turbidity [146]. Although such a wide array of assays exist, this is the only assay that targets mDNA and combines calcein with real-time detection instrumentation.

5.4.4 Limitations of the in-house assay

Despite the considerable advances made in the development of an in-house LAMP assay, further obstacles remain which are likely to impede field utilization in its current form. The in-house assay cannot currently be utilized as a field test as all reagents are stored at -20 °C. Such storage requires specialized and expensive freezers that are often not available at centralized hospitals, let alone in peripheral health care establishments. LAMP reagents can be lyophilized as demonstrated by the Eiken kits, so it may be possible to do the same with the in-house assay but this was outside the scope of this project.

To increase ease of use of the in-house assay, sample processing and subsequent LAMP testing would be, ideally, condensed into a single step. The current workflow of LAMP requires many steps including multiple transfer steps, heating, centrifuging, and interpreting results under specific conditions with specialized equipment. To truly make the assay accessible to remote communities, this workflow needs to be significantly simplified. Ideally, DNA extraction should either be omitted or occur simultaneously with the LAMP assay and the assay itself would be more streamlined with less manual handling steps to match the simplicity of a test like the RDT. **Chapter 6** explores this ideology further by combining sample preparation and LAMP incubation into one step.

When the in-house LAMP assay is assessed under the ASSURED framework, it meets most criteria. In its current format, it is Affordable (**Section 5.3.4**), Sensitive and Specific (**Section 5.3.3**), and Rapid and robust (**Section 5.3.2**). Although not Equipment- free, the use of instrumentation increases its performance in all other criteria (real-time detection enables rapidity, sensitivity, and specificity). Improvements can still be made in the User-friendly and Deliverable to end-user categories which will be addressed in subsequent chapters of this thesis.

5.1 Conclusion

An in-house assay was developed as a cheaper alternative to the Eiken kits as a diagnostic test for malaria. Areas such as reagent concentration, incubation temperature, and primer target were all investigated to make the most sensitive assay possible. The importance of instrumentation was discussed in regards to interpreting results accurately and alternative simpler DNA extraction methods were explored. While the in-house assay was very sensitive and easy to use, the wet chemistry format of the assay limits it use in field settings and extensive manual handling is still required for DNA extraction. Future work will look into integrating DNA and LAMP incubation into one step, and stabilising the LAMP reagents to allow for easier storage and assay assembly.

6 Increasing field applicability of malaria LAMP assay using the T1-ISO platform

6.1 Introduction

To successfully eliminate malaria, there is a need for high-sensitivity testing that can be deployed in remote and resource-constrained field settings, with workflows that are suitable for end-users with minimal training, and which have a short time from sampling to result. The advantages of LAMP over other malaria diagnostic tests for this type of application have been explored throughout this thesis. While LAMP has the potential to be used as a routine high-sensitivity screening tool in elimination settings, there are implementation challenges that will need to be overcome if it is to be successfully deployed.

In the current format, the assay is a three-step process of DNA extraction, LAMP amplification and detection. The need to simplify this workflow has been highlighted by several studies [48, 147]. When implemented in field settings, operators have found DNA extraction to be the most labour-intensive aspect of the assay, especially when large numbers of samples are to be tested [148]. Several studies have identified that simplified DNA extraction methods are critical and that these methods also need to be rapid, cost effective, and to produce high yields of template DNA [149, 150]. LAMP already provides potential benefits over PCR in this regard, with many studies showing that *Bst* polymerase is more tolerant to the inhibitors found in blood (e.g. haem, IgG, and IgM) than the *Taq* polymerase used in most PCR reactions [151-153]. DNA extraction, even with the simplified approaches identified in **Chapter 4**, requires a heating step and in some instances a centrifugation step. Simplifying this DNA extraction process further, and combining it with LAMP amplification and detection, could lead to successful implementation of LAMP in field settings. In this chapter simplifying this workflow for the end-user is explored using a cartridge and instrument platform, called T1-ISO, that is in development with our commercial collaborator, Axxin Pty Ltd (Fairfield, Australia).

The capacity of multiplexing LAMP is also explored using a melt-curve analysis approach. This has the potential to improve diagnostics by differentiating different species based on different amplicon binding characteristics. In malaria positive cases, the species could potentially be identified immediately, without requiring subsequent species-specific LAMP testing or the need to multiplex using molecular probes or spatially-separated LAMP reactions. Species-specific LAMP would have a

dramatic impact on informing appropriate treatment [154, 155]. These approaches could also be used to combine non-*Plasmodium* targets. Therefore, symptomatic patients with an undefined febrile illnesses, that test negative for malaria, could have a diagnosis made simultaneously for other key infections [156, 157]. Combining multiple tests into one could also be more affordable for health care providers [158]. From a research perspective, collecting data on multiple diseases at once could extend resources and be less laborious, and insights of how diseases are interacting with each other might also become more apparent. A specially modified isothermal instrument was developed by Axxin Pty Ltd for this part of the project to assess the utility of melt-curve analysis for these applications.

This chapter undertakes early stage development of a simplified malaria LAMP workflow using an integrated cartridge and instrument platform. It assesses a range of assay-cartridge-instrument integration issues including: optimising blood and lysis buffer volumes, dispensing volumes from the sample processing chamber into the LAMP reaction chamber, buffer compatibility (i.e. sample lysis buffer with LAMP reaction conditions), and an early assessment of assay sensitivity. The potential for melt-curve analysis to multiplex the LAMP reaction was also investigated.

6.2 Methods

The T1-ISO platform

The T1-ISO platform (**Figure 6.1 A**) designed by Axxin Pty Ltd is an isothermal cartridge and instrument that can be used to integrate sample processing and LAMP testing. The T1 cartridge (**Figure 6.1 B**) was designed for testing of one clinical sample at a time in a completely closed system. This helps to minimise infectious risk to the user, and decrease the potential for cross-contamination. This platform has limited throughput, but in this instance was used as a development platform that may be adapted in the future for high-throughput platform suitable for population-based screening.

The T1 platform cartridge and instrument were used in this project to develop a malaria LAMP, using the findings from **Chapter 5** of this thesis. In broad terms, the workflow involved addition of whole blood to the sample processing chamber which contained a sample lysis buffer. After sample lysis, the plunger was inserted into the sample processing chamber and the attached lid screwed into the body of

the chamber. In this form of the cartridge, there was deliberately no DNA purification step. This was to assess the potential for extremely low-cost sample preparation. When the lid was screwed down, the piercing rod was able to puncture the membrane between the sample processing chamber and the LAMP reaction tube below. A 90-100 µl volume of the lysed sample was then dispensed into the LAMP reaction tube, where the LAMP reagents were already contained. The dispensed volume is determined by the dispense reservoir and can be designed to dispense different volumes. After being dispensed, the LAMP amplification and real-time fluorescent detection was then able to proceed.

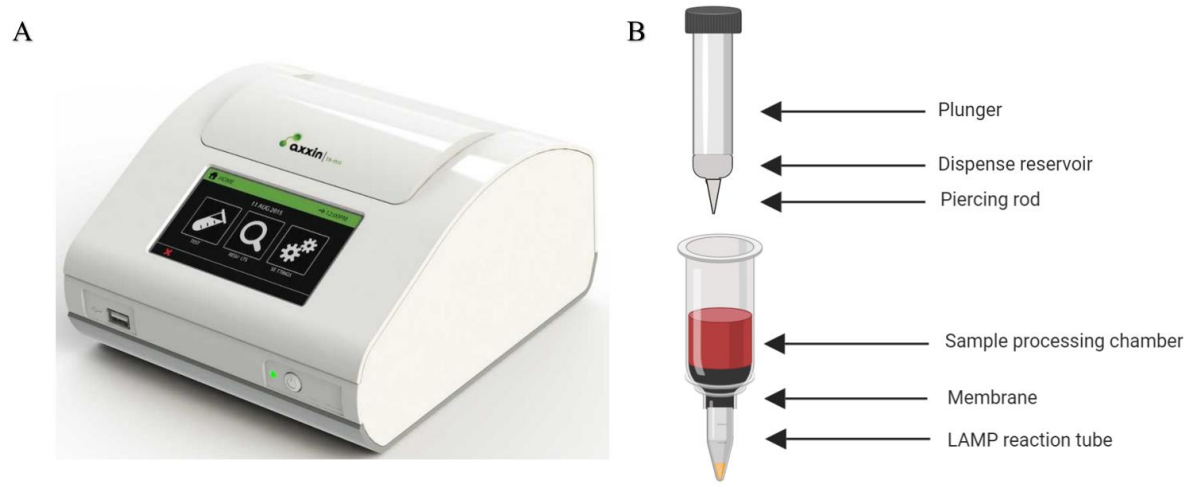

Figure 6.1: The T1-ISO platform

(A) The T1-ISO instrument designed by Axxin. The LCD user interface provides a prompt for each of the workflow steps and displays automated results. **(B)** T1 cartridge designed by Axxin. Sample lysis and DNA extraction occurs in the sample processing chamber, the piercing rod punctures membrane dispensing the set volume in the reservoir into the LAMP reaction tube below.

6.2.1 Combining sample preparation and LAMP testing in T1

Determining the minimum capacity of the sample processing chamber

These experiments were designed to determine if the volume of the buffer in the sample processing chamber affected the volume dispensed into the amplification tube of the T1 cartridge. Volumes of lysis buffer (10 mM Tris pH 7.8, 0.4% Triton X, 0.4% saponin) ranging from 500 µl to 2000 µl were tested in the T1 cartridge. Lysis buffer was added to the sample processing chamber and dispensed by screwing the plunger with the piercing rod attached until it punctured the membrane. Each volume was assessed using six replicates. The volume and weight of the dispensed buffer was recorded and the accuracy and repeatability of each volume was assessed.

Assessing the impact of LAMP reagent volume on dispense volume

These experiments were designed to determine if a liquid volume in the amplification tube (below) would affect the dispensed volume from the sample processing chamber (above) of the T1 cartridge (i.e. the impact of dispensing into a tube with decreased liquid-free volume). Volumes of lysis buffer ranging from 500 µl to 2000 µl were used in the T1 cartridge to dispense into LAMP reaction tubes with or without a volume of 100 µl of distilled water in the tube. Each condition was assessed using six replicates. The volume and weight of the dispensed buffer was recorded and the accuracy and repeatability of each volume was assessed.

Determining the maximum volume of lysed blood feasible for LAMP processing

These experiments were designed to assess the optimal volume of the lysed sample to be dispensed from the sample processing chamber into the LAMP reaction tube. Five microlitres of *P. falciparum*-infected blood (concentration of 50,000 parasites/µl from *in vitro* parasite culture) was added to 45 µl of lysis buffer. Increasing volumes of this lysed blood, from 2 to 9 µl, were added to the LAMP reaction. Water volume was adjusted to maintain a 25 µl total LAMP reaction volume. It should be noted that while the final cartridge would ideally have dried LAMP reagents, the intention of these experiments was to get a preliminary insight into relative wet volumes of lysed sample and LAMP volumes. These assays were run in the T16 isothermal instrument (as described in **Section 2.4.2**).

Determining the maximum sample volume feasible for lysis

These experiments were designed to assess the optimal volumes of blood and sample lysis buffer. Combinations of 50 µl and 100 µl *P. falciparum* infected whole blood sample (concentration of 50,000 parasites/µl from *in vitro* parasite culture) were added to 1000 µl and 2000 µl of sample lysis buffer. Eighty microliters of each condition were added to 142 µl of LAMP buffer. Reactions were run in the T16 isothermal instrument (as described in **Section 2.4.2**).

Combining lysis and LAMP in T1 cartridge

Once the optimal volumes and ratios of sample to lysis buffer and lysed blood to LAMP reagents ratios were identified, this method was adapted to the T1 cartridge. Test samples were thoroughly mixed into the lysis buffer by physically inverting the tubes prior to dispensing. Once the sample was dispensed, T1 cartridges were either run in the T1 or the LAMP reaction tube was removed and run in the T16 isothermal instrument if higher throughput was required.

Determining the sensitivity of the T1 system

Using the best performing ratios for sample to lysis buffer and lysed blood to LAMP reaction, the sensitivity of the T1 system was determined by using a titration of *in vitro* cultured *P. falciparum* parasites (i.e. 3D7 laboratory line) with parasite densities ranging from 50,000 parasites/µl to 0.5 parasites/µl. Reactions were run in the T1 with the same conditions as in the T16 isothermal instrument.

6.2.2 Multiplexing LAMP reactions using melt curve analysis

SYTO-82 as a detection modality for LAMP reactions

SYTO-82 (instead of calcein which was used in **Chapter 5** of this thesis) was used for detection of LAMP reactions that underwent melt curve analysis, because melt-curve analysis required the use of either an intercalating dye or integrated fluorescent probe. The non-specific mechanism of calcein detection would not have been appropriate for melt-curve analysis. SYTO-82 was used at a final concentration of 2 µM and fresh working stocks were prepared before each run. Amplification was detected in the HEX channel at 18% gain (intensity of LED excitation). All other LAMP reagents remained unchanged.

Melt curve analysis of isothermal products in the T8-ISO

Melt curve analysis was set up in a specially modified T8-ISO isothermal instrument. As dsDNA denatured becoming ssDNA, the SYTO-82 is predicted to dissociate from the DNA, thereby decreasing fluorescence of the sample. Amplicons with lower dissociation temperature would be expected to be differentiated from amplicons with higher dissociation temperatures. Melt curve analysis was performed over a temperature range of 60 °C to 95 °C. Temperature was increased incrementally by 0.2 °C where it held for 12 seconds and a reading was obtained. The total run time for the melt curve analysis was 35 minutes. Melt curve analysis was used to distinguish between non-specific amplification, and amplicons from different species (i.e. *P. falciparum* and *P. vivax*) and different gene targets (i.e. 18S and mitochondrial).

6.3 Results

6.3.1 Combining sample preparation and LAMP testing in T1

The volume of lysis buffer in the sample processing chamber affected the volume dispensed into the LAMP tube

The sample processing chamber has a maximum sample lysis buffer capacity of 4 ml. However, use of a smaller volume would save on reagent costs and would potentially decrease the sample volume required for an optimal sample to lysis buffer ratio (i.e. would not affect sensitivity by diluting sample). It is intended that this system would use a finger prick sample, which usually ranges between 50-100

µl (based on sampling methods used in the RAI study described in **Chapter 4**). Results indicated that there was a minimum sample buffer volume required in the sample processing to ensure that the dispense reservoir would be completely filled and that an accurate volume of buffer was dispensed into the LAMP tube below. Of the volumes of lysis buffer trialled, 500 µl was not sufficient to completely fill the dispense reservoir (**Figure 6.2**). Volumes of 1000 µl and greater were sufficient to fill the dispense reservoir on visual inspection.

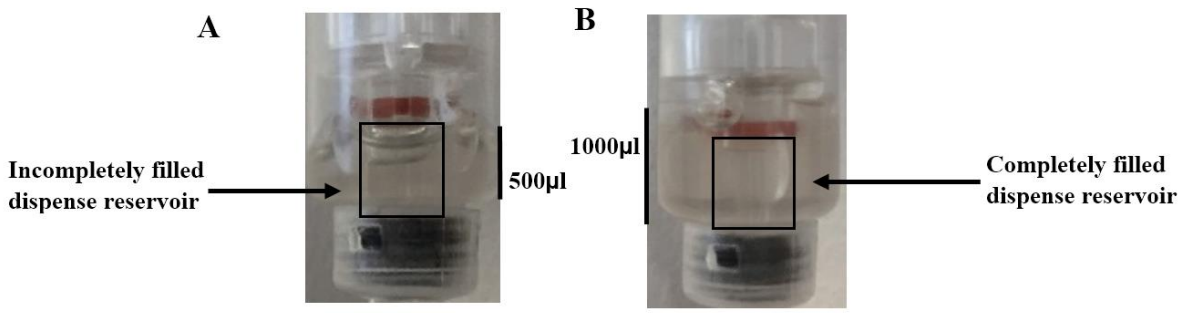

Figure 6.2: Minimum lysis buffer required in sample processing chamber.

The dispense reservoir in **(A)** did not fill completely with 500 µl of lysis buffer. A volume of 1000 µl **(B)** was sufficient for the dispense reservoir to be completely filled.

Figure 6.3 shows the average dispense volume of each lysis buffer volume from six replicate samples. The average dispense volumes were as follows; 86 µl with 500 µl lysis buffer, 91 µl with 1000 µl lysis buffer, 101 µl with 1500 µl lysis buffer, and 88 µl with 2000 µl. Unpaired t-tests were used to compare each lysis buffer volume to each other and statistical significance was only seen between 500 µl and 1500 µl lysis buffer volumes (p=0.019) The largest and least variable average dispense volume was seen with 1500 µl lysis buffer (mean dispense volume=101 µl; range=66-104 µl; standard deviation 7.9 µl; n=6). Although the 1500 µl lysis buffer dispenses were less variable than those seen in the 1000 µl condition (SD=7.9 compared to SD=13.8 respectively), 1000 µl was used in subsequent tests. This was to ensure that the lysis volume was as small as possible to avoid diluting the sample and to maximise the amount of sample dispensed into the LAMP reaction.

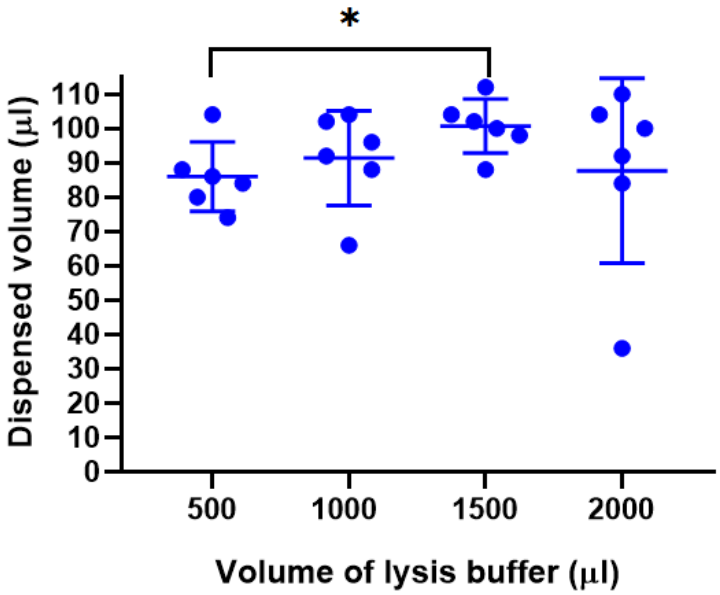

Figure 6.3: Average dispense volume when varying lysis buffer volumes.
Lysis buffer volumes of 500 µl, 1000 µl, 1500 µl, and 2000 µl were trialled in the T1 cartridge. The average dispense volume into empty test tubes over six replicates of each lysis buffer volume is shown. The largest average volume dispensed was seen with 1500 µl lysis buffer. Unpaired t-tests were used to assess statistical significance. Mean and standard deviation are shown.

Dispense mechanism is compatible with filled LAMP reaction tube

In the T1 cartridge, when lysed blood is dispensed from the sample processing chamber, air in the LAMP reaction tube below is displaced to accommodate the addition of this fluid volume. Dispensing into a LAMP reaction tube with liquid volume already in the tube, would potentially impact on the accuracy of the dispensed volume. The effect that this would have on the repeatability and accuracy of the dispense volume was assessed. When comparing the volume of buffer dispensed into an empty LAMP tube with the volume dispensed into a tube with 100 µl of liquid already in it (**Figure 6.4**), the dispense volume decreased slightly for all volumes tested. The average dispense volumes were as follows; 80 µl with 500 µl lysis buffer, 86 µl with 1000 µl lysis buffer, 79 µl with 1500 µl lysis buffer, and 84 µl with 2000 µl. The difference between empty and liquid-containing LAMP reaction tubes was only statistically different when using 1500 µl lysis buffer (p=0.008). The largest average dispense volume was achieved with 1000 µl lysis buffer (mean dispense volume=86 µl; range=74-102 µl; standard deviation=10.5 µl; n=6), thus was used in subsequent experiments.

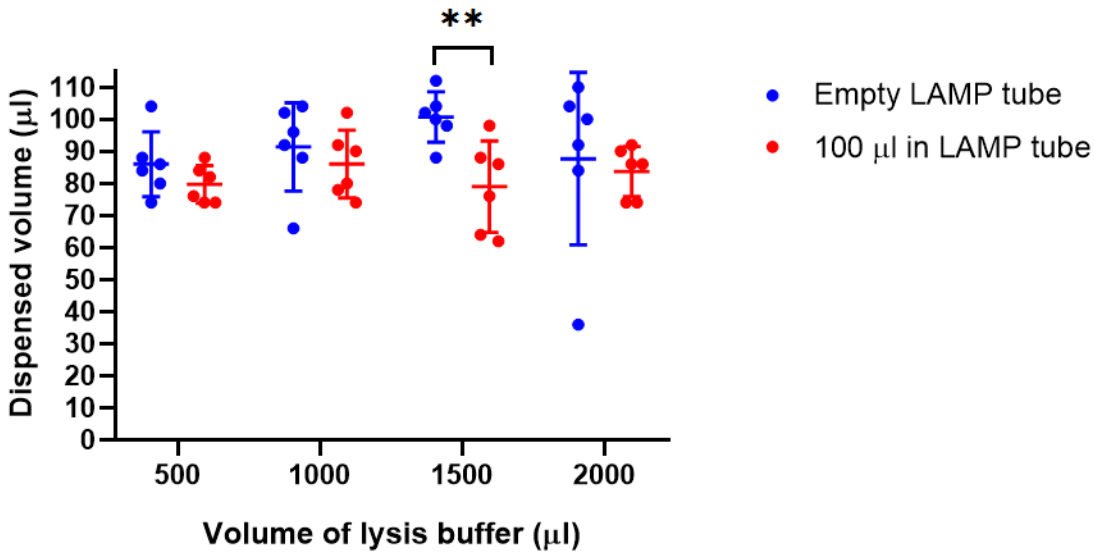

Figure 6.4: Average dispense volumes with fluid volume of reagents in LAMP tube
Volumes of 500 µl, 1000 µl, 1500 µl, and 2000 µl were trialled in the T1 cartridge. The average dispense volume into test tubes containing 100 µl of LAMP reagents over six replicates of each lysis buffer volume is shown. The largest average volume dispensed was seen with 1000 µl lysis buffer with an average of 86 µl. Statistical significance determined using the Holm-Sidak method. Each row was analysed individually, without assuming a consistent SD. Mean and standard deviation are shown.

LAMP is tolerant to large volumes of un-purified lysed blood

An important way to maximise sensitivity involves increasing the volume of lysed blood added to the LAMP reaction. Increasing the volume of lysed blood may affect the LAMP reaction by altering the pH, increase the presence of inhibitory compounds, or may increase the amount of discoloration in the LAMP reaction mostly from haemoglobin. To assess this, the maximum amount of lysed blood that could be added to the LAMP reaction, without compromising sensitivity, was determined for amplification efficiency. A decrease was seen in the fluorescent signal of reactions with lysed blood added compared to positive and negative controls (purified genomic DNA or water respectively) and this difference increased with increasing lysed blood volume (i.e. fluorescence was more impaired in 9 µl of lysed blood compared to 2 µl) (**Figure 6.5**). The time to amplification of lysed blood reactions compared to control reactions was delayed, but no difference was seen with increasing lysed blood volumes. This implies that 9 µl of lysed blood (for every 25 µl reaction), does not considerably impact the LAMP reaction. This ratio of 9 µl lysed blood to 16 µl LAMP reagents (all reagents except water) was used as the basis of subsequent experiments. When volumes were scaled up for use in the T1 cartridge, this ratio was maintained as closely as possible.

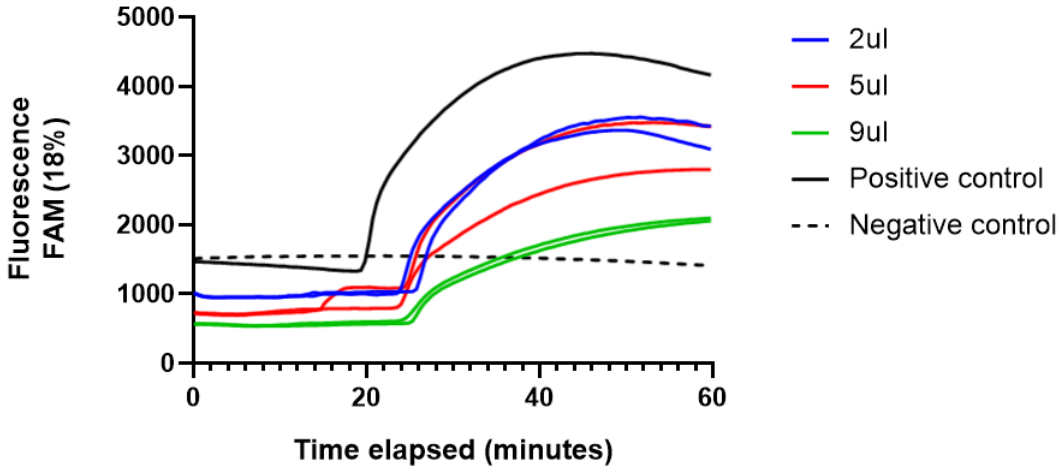

Figure 6.5: Impact of lysed blood volume on LAMP amplification.
Varying volumes of lysed blood were added to the LAMP reaction. Extra volume of lysed blood was accounted for by decreasing the water volume in the LAMP reaction (2 µl lysed blood had 7 µl of water in the reaction, 5µl of lysed blood had 4 µl of water and 9µl of lysed blood had no water added). No difference in time to amplification was seen between conditions however decreased fluorescent signals were seen in the test conditions compared to the positive control. These experiments were performed on the T16-ISO instrument to enable higher throughput assessment. The positive control was purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage

Sensitivity of LAMP detection was not inhibited by pigmented lysed blood

The tolerance of LAMP to biological inhibitors has been demonstrated in the wider literature and in **Chapter 4** where the simple boil and spin extraction method was compatible with the Eiken LAMP kits. Cell lysis is an essential step in DNA extraction protocols and subsequent steps involve purifying the DNA. As LAMP is tolerant to inhibitors, there may be less of a need for purification and direct lysis alone may be sufficient. To achieve sufficient sensitivity (especially with samples of low parasite density) a large enough blood volume needs to be included in this lysis step. However, as shown above, blood samples are highly pigmented and this can impact on the overall fluorescence signal during the amplification process. Each of the conditions studied resulted in similar time of amplification and each had a clear increase in fluorescence from their respective baselines (**Figure 6.6**). Using 50 µl of blood in 1000 µl of lysis buffer (with an 80 µl dispense volume) would provide a good balance between a feasible finger prick blood volume and high sensitivity by having a high ratio of blood to lysis buffer, and higher ratio of dispense volume relative to the overall lysis buffer volume.

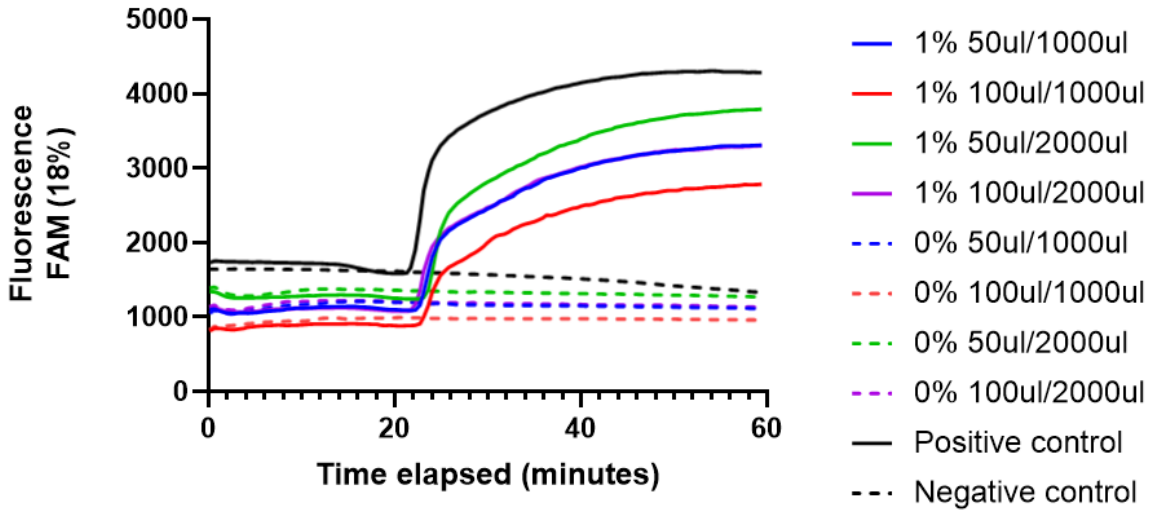

Figure 6.6: Effect of sample volume on LAMP amplification

Combinations of 50 µl and 100 µl sample (50,000 parasites/µl) and 1000 µl or 2000 µl lysis buffer were trialled in the LAMP reaction. Amplification was not substantially impacted by the lysed blood demonstrated by only a slight delay in time to amplification as compared to the positive control. The combination deemed most suitable was 50 µl of sample and 1000 µl of lysis buffer. These experiments were performed in the T16-ISO instrument increase throughput. Each sample was tested in single replicates. The positive control was purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage.

Combining sample lysis and LAMP in T1 cartridge

Four important aspects of the T1 cartridge were assessed: (1) the minimum volume of lysis buffer volume required, (2) the average dispense volume, (3) the maximum volume of lysed blood compatible with the LAMP reagents, and (4) the maximum lysed blood volume dispensed into the LAMP tube without detection inhibition. The complete workflow was then translated into the T1 cartridge and the T1 isothermal instrument. **Figure 6.7** shows the workflow of the T1 from sample preparation to amplification in the T1-ISO. Amplification proceeds in the same way that it does in other isothermal platforms, but the settling of the blood products (as they coagulate with the heat and become heavier) interferes with the detection of calcein initially. A strong fluorescent signal can then be seen after the blood products settle completely just before 30 minutes. Earlier versions of the T1 amplification test type included heating the sample processing chamber, but this was deemed to be unnecessary and so was not used in subsequent versions.

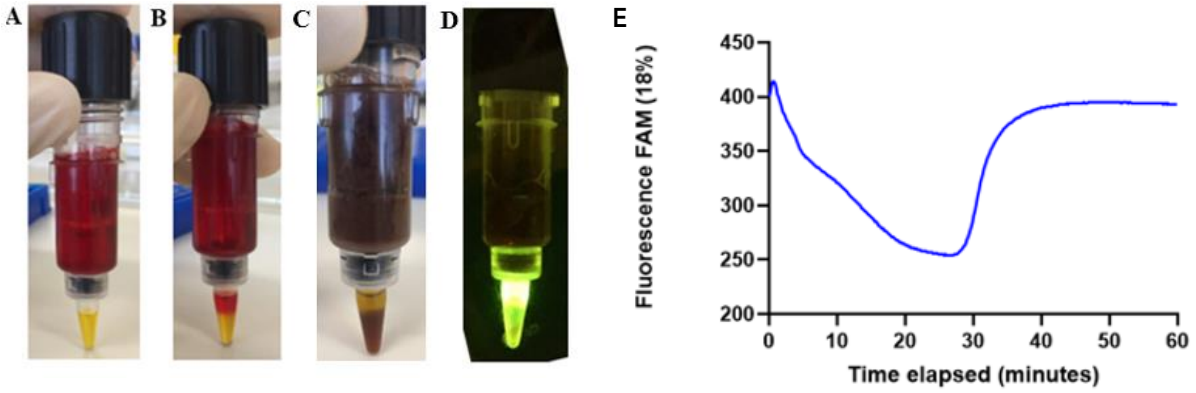

Figure 6.7: Sample processing and LAMP in T1 cartridge.
(A) A T1 cartridge with lysed blood (and lysis buffer) in the sample processing chamber connected to LAMP reagents in the LAMP reaction tube below, **(B)** the cartridge immediately after lysed blood has been dispensed in to the LAMP reaction tube, **(C)** after LAMP amplification in white light and **(D)** under UV light. **(E)** T1 fluorescent output (read in the FAM channel of the T1-ISO instrument) of amplification. The sample lysed was whole blood spiked with 50,000 parasites/µl.

Determining the sensitivity of the T1 system.

Due to time constraints the sensitivity of this workflow was not assessed. Future work will combine all optimised aspects of the workflow and test it over a range of parasite densities to evaluate the LOD of this workflow.

6.3.2 Multiplexing LAMP reaction with melt-curve analysis

SYTO-82 as a detection modality for LAMP reactions

SYTO-82 is a dye that intercalates into dsDNA during the amplification process [159]. Unlike calcein, which detects by-products of amplification, SYTO-82 can be used in melt curve analysis because of its intercalation with dsDNA.

Melt curve analysis of isothermal products in the T8-ISO

Melt curve analysis was performed immediately after the LAMP amplification step. This work was conducted on a version of Axxin's T8 isothermal instrument that was specially customised for this project.

Replicate samples differ slightly in fluorescent intensity due to optical efficiency differences between samples [160], so curves were normalized to make melting transitions easier to compare. Curves were then plotted as negative first derivative plots showing curve peaks as approximate melting temperatures. This graphical transition can be seen in **Figure 6.8**.

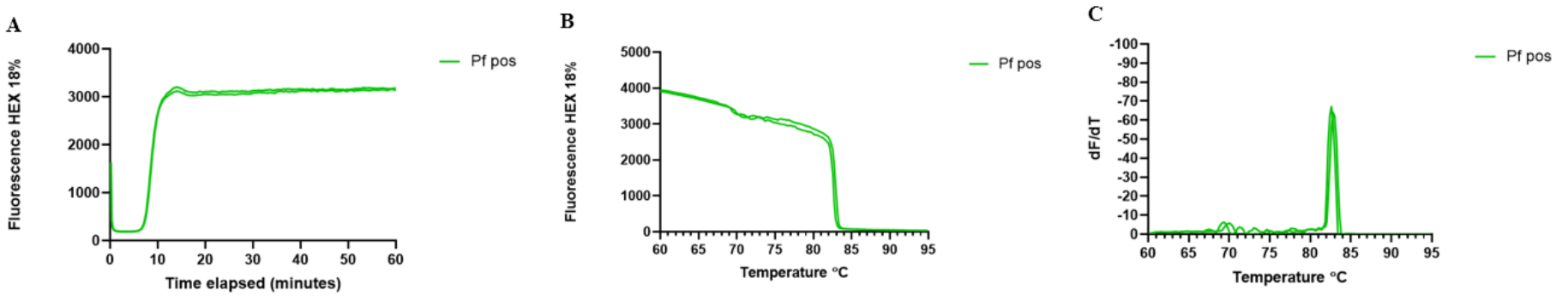

Figure 6.8: Example of an amplification curve and melt curve analysis of a positive malaria sample.

(A) Amplification of *P. falciparum* DNA using specific mDNA primer targets over 60 minutes with fluorescence detected in the HEX channel, **(B)** melt curve analysis of resulting amplicons showing a decrease in detectable fluorescence once dsDNA dissociates, and **(C)** the negative first derivative of the melt curve showing the melting temperature of this product to be 83 °C. The Y-axis in this case represents the negative derivative of fluorescence over time. The positive control was purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage, performed in duplicate.

Melt curve analysis of non-specific amplification

Melt curve analysis had varying capacity to discern between true amplification and non-specific amplification. A clear difference was seen in *P. vivax* samples where the melt profile of non-specific amplification were present as high peaks between 85-87 °C and true amplification was seen as a double dissociation curve (**Figure 6.9 A**). Less of a difference was so in *P. falciparum* samples. The different primer targets for *P. falciparum* have a similar melting temperature of 83.4 °C (18S rRNA) and 82.7°C (mDNA) but can be distinguished by the increased fluorescence signal (resulting in a higher peak for mDNA targets) (**Figure 6.9 B** and **C**).

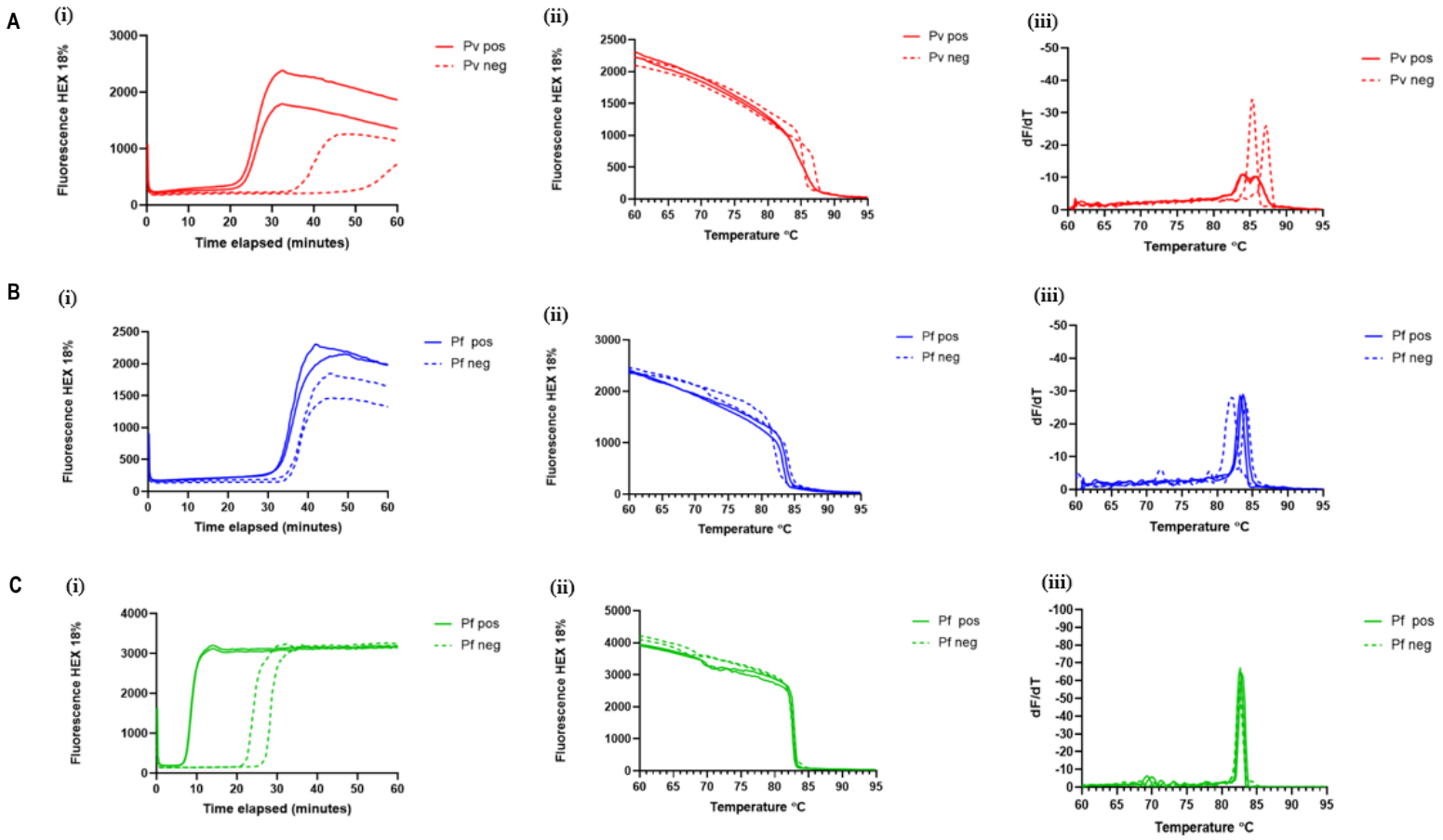

Figure 6.9: Melt curve analysis of true positives and non-specific amplification.
Melt curve profiles for true positives and non-specific amplification were compared. Melt curves of positive and negative samples show clear differences in non-specific amplification of *P. vivax* samples amplified using the 18S rRNA target **(A)**, less of a difference can be seen in *P. falciparum* samples amplified using the 18S rRNA target **(B)** and no difference can be seen in mDNA targeted *P. falciparum* samples **(C)**. **(i)** Amplification curve in T16-ISO, **(ii)** melt curve in T8-ISO, and **(iii)** negative first derivative plots. *P. vivax* positive control was extracted DNA from a reference sample with $2x10^6$ copies of the 18S rRNA target, *P. falciparum* positive control was either extracted DNA from a reference sample with $2x10^6$ copies of the 18S rRNA target **(B)**, or purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage **(C)**. Negative controls were NFW in all cases. All samples were performed in duplicate.

Melt curve analysis to distinguish between Plasmodium species

Typical curves for *P. falciparum* and *P. vivax* infections were distinct, allowing clear discrimination between species. A mixed infection (i.e. *P. falciparum* and *P. vivax*) was also distinct from each single *P. falciparum* or *P. vivax* infection. Using LAMP primers targeting species specific 18S rRNA targets, the melting temperature of *P. falciparum* amplicons was 83.4 °C. This differed greatly from the double dissociation curve seen for *P. vivax* 18S rRNA amplicons which has maxima at 84.0 °C and 85.6 °C for *P. vivax* amplicons (**Figure 6.10 A**). When *Plasmodium* genus primers were used (which targeted the conserved 18S rRNA regions of *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P knowlesi*) to amplify *P. falciparum* and *P. vivax* samples in one reaction, the precise melting temperature for the dual infection is approximately 84.9 °C. However distinct peaks were not seen for each species and the curve profile was very similar to *P. falciparum* reactions (**Figure 6.10 B**). When *P. falciparum* and *P. vivax* primers were amplified together however (half the amount of each species specific primer set and half the amount of each species specific template), a curve profile and melting temperature distinct from both individual *P. falciparum* and *P. vivax* reactions was evident (**Figure 6.10 C**).

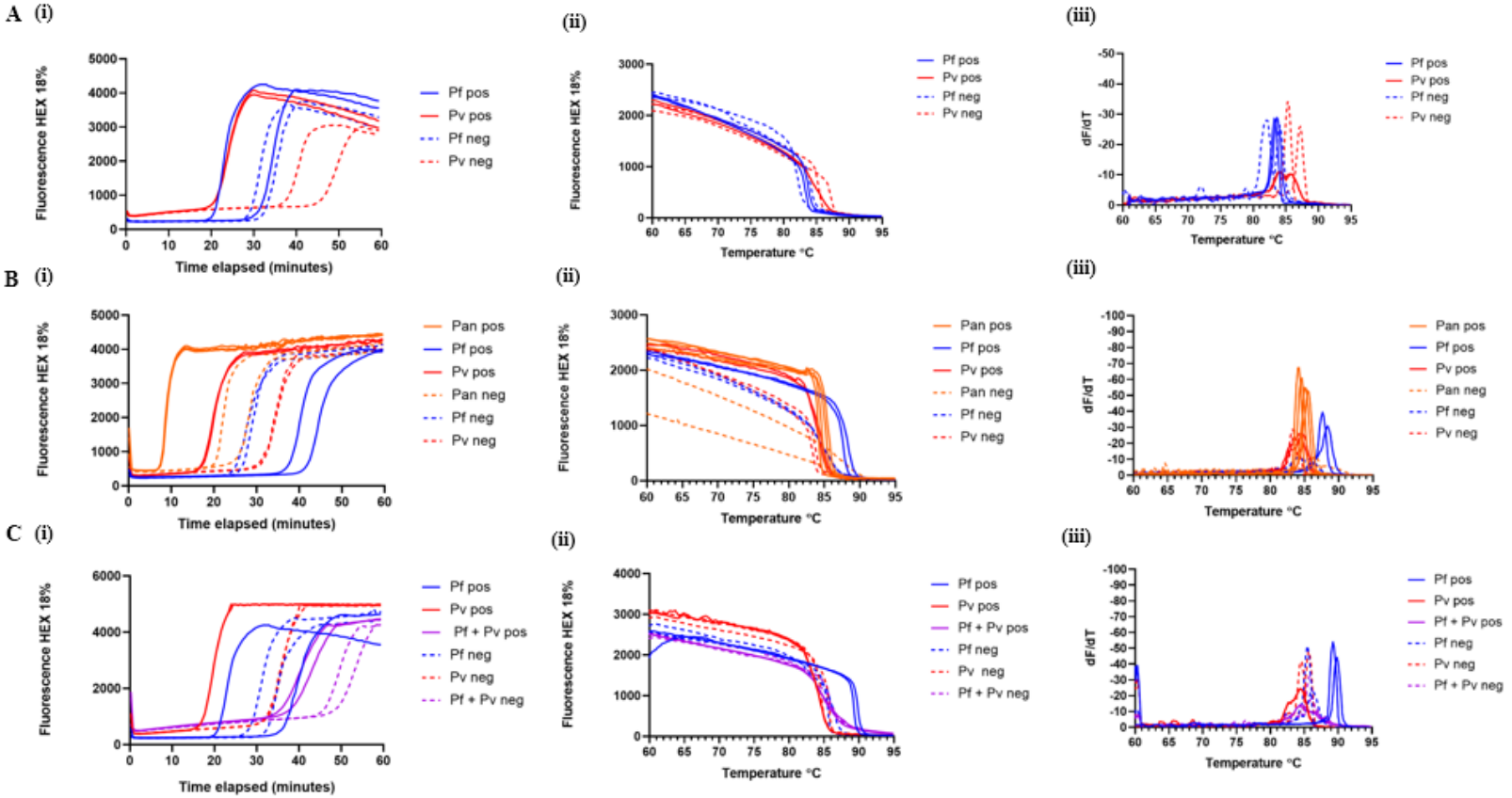

Figure 6.10: Melt curve analysis of different Plasmodium species and mixed infections.

Melt curve profiles of different Plasmodium species in single and mixed infections were compared. **(A)** Distinct differences can be seen in the curve shape and melting temperature of *P. falciparum* and *P. vivax* positive samples with *P. falciparum* samples having a peak melting temperature of 88 °C and *P. vivax* having two peaks at 84 °C and 86 °C**. (B)** When a sample was amplified with *Plasmodium* genus primers, another curve is seen which resides between the curves of single *P. falciparum* and *P. vivax* positive samples, however individual curves are not seen for each species. When both species specific primer sets are used a more distinct curve is apparent (**C**). **(i)** Amplification curve in T16-ISO, **(ii)** melt curve in T8-ISO, and **(iii)** negative first derivative plots 18S rRNA targeting primers were used in all cases. Positive controls were species specific extracted DNA from a reference sample with $2x10^6$ copies of the 18S rRNA target. For single reactions (**A**) 4 µl of positive control was used and for mixed reactions (**B and C**) 2 µl of *P. falciparum* and 2 µl of *P .vivax* positive control were used. NFW was used for all negative controls. All samples were performed in duplicate.

Melt curve analysis to distinguish between primer targets

Different primer targets are also readily distinguishable using melt curve analysis. Time to amplification is much slower when using 18S rRNA targeted primers (20-30 minutes) compared to mDNA targeted primers (8 minutes). Differences in the melt curve profile can also be seen with mDNA positive samples having a tall peak at 81.9 °C and 18S rRNA samples having a shorter peak at 83.3 °C (**Figure 6.11**).

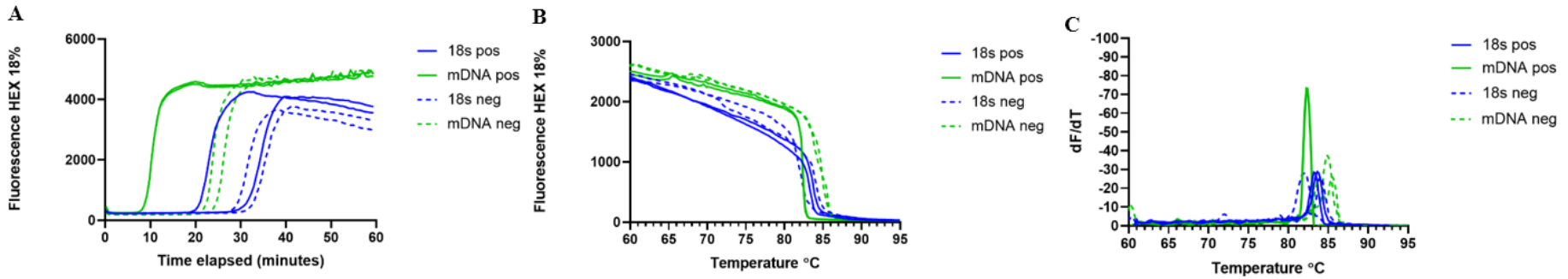

Figure 6.11: Melt curve analysis of different primer targets for P. falciparum amplification

Melt curve profiles of different *P. falciparum* primer targets were compared. **(A)** Amplification curve in T16-ISO, **(B)** melt curve in T8-ISO, and **(C)** negative first derivative plots for 18S rRNA and mDNA targeting primers. mDNA primer yield a faster time to amplification than 18S rRNA primers, and distinct melt curves are produced for both targets. Positive controls for 18S rRNA samples were extracted DNA from a reference sample with $2x10^6$ copies of the 18S rRNA target and for mDNA were purified genomic DNA extracted from cultured 3D7 laboratory strain *P. falciparum* parasites at a parasitaemia of 4% ring stage. NFW was used for all negative controls. All samples were performed in duplicate.

6.4 Discussion

The T1 workflow can improve the field practicality of LAMP assays

The research presented in this chapter demonstrated proof of principle on the T1-ISO platform for simplified workflow for sample processing, LAMP amplification and detection. All components of the workflow were optimised separately before combining into an integrated cartridge. An acceptable amount of fluorescent signal inhibition was observed, although variability of dispense volumes could be improved. The sensitivity of the T1 workflow was not assessed due to time constraints however, the T1 sample processing workflow was much simpler than traditional DNA extraction methodologies, and was not reliant on equipment making it ideal for use in resource limited settings.

Further improvements on the dispense volume of the T1 cartridge could also be made. In the current iteration, the variability in the dispense volume of the T1 cartridge may have downstream effects on sensitivity especially at lower parasite densities. Replacing the thin plastic membrane between the sample processing chamber and the LAMP reaction tube with a material more easily punctured (such as a reinforced foil-type material) may address this issue. Collaborative efforts are currently underway with Axxin to develop viable options.

The T1 platform provides a convenient system to test assay integration, but the real need in malaria elimination settings is for higher throughput of sample testing [43, 161]. This could be addressed with multiple LAMP reaction tubes connected to the sample processing chamber and incubation occurring in an isothermal instrument with a greater number of tubes. While design modifications will be necessary, such a cartridge is highly feasible. The preliminary data presented here provides a solid proof of concept to undertake future development of a high-throughput isothermal platform.

The assay development conducted on the T1 need not be confined to a single sample processing unit. The chemistry can be applied in any format including multi-chamber microfluidic type cartridges. These formats have been trialled with LAMP [162-164], and integration of the T1 sample processing methodology is feasible. Because no heating or centrifugation is required, few modifications to the assay workflow or reagent composition will be necessary. Axxin manufacture lateral flow readers, enabling the same level of data acquisition and analysis used in the T1-ISO or T8-ISO platforms.

Evolving this chemistry beyond one sample preparation will allow the processing of multiple samples at once, increasing throughput while decreasing time expended.

Lyophilising LAMP reagents in the reaction tube would improve test stability and possibly increase sensitivity [165, 166]. LAMP reagents are more stable and do not require refrigeration if they are lyophilised. The commercial LAMP assay from Eiken has adopted this approach, and as such their kits have a long shelf life at room temperature (albeit still being susceptible to extremes in temperature and humidity). Appropriate cryoprotectants that preserve the functionality of the dried reagents without inhibiting the LAMP amplification are essential [167, 168]; and a matrix excipient or caking agent may also be appropriate. LAMP reactions using glycerol-containing *Bst* enzymes and betaine may prevent complete drying [169], so can be substituted for similar reagents. Lyophilising LAMP reagents would improve stability and ease of use, and would increase the amount of available template in a reaction as more sample could be added.

Multiplexing LAMP using melt-curve analysis has numerous applications

The potential inclusion of melt-cure analysis on LAMP amplified products was demonstrated. SYTO-82 was successfully used to detect LAMP amplification and then to subsequently perform melt curve analysis on amplicons produced. In collaboration with Axxin Pty Ltd, an isothermal instrument was specially customized and melt curve protocol was optimized for the LAMP reaction. These data represent the first data from this prototype instrument, and demonstrated that different *Plasmodium* species (and mixed infections) yielded different melt curve profiles. The curves differed in the peak melting temperature, but also in height, width, and overall appearance. They could be used to determine the causative species of an infection (which *Plasmodium* species, single or multiple infection), the difference between true positives and amplification artefacts, and between different primer sets targeting the same pathogen. At the time of writing and to the best of our knowledge, performing melt curve analysis with LAMP for *Plasmodium* detection and speciation has only been demonstrated once before [170].

Treatment is not usually administered until the *Plasmodium* species has been identified as treatment regimes may differ. Participants in the pilot study described in **Chapter 4** were only screened for the *Plasmodium* genus. Other versions of the commercial LAMP assay do test specifically for *P. falciparum* and *P. vivax*, but this requires an additional two tests, usually conducted after a positive *Plasmodium* genus LAMP result. This delays the diagnostic pathway and time to treatment, increases cost, and may not be feasible when screening mobile populations. Performing melt curve analysis on LAMP positive samples may allow for each species to be identified, using their different temperature melting profiles without performing any additional tests.

Screening all *Plasmodium* species (and combinations thereof) will be important to assemble a catalogue that could be used as a reference when screening clinical samples. To ensure accuracy of the profiles, a large number of samples would be required for this purpose to capture the variation between clinical isolates [171]. Multiple infections detected by *Plasmodium* genus primers did not yield individual peaks for each species (i.e. all amplicons included in one melt curve); however, a distinct peak was seen when species specific primers were combined. Further work will test combinations of primer sets to determine typical melt curve profiles for mixed infections. Labelling LAMP primers with fluorescent probes would also enable speciation [172, 173], but such methods would require extensive optimization.

Slight variations in the melt curve patterns and peak temperatures between experiments are common. Slight shifts in melting temperatures are often seen, but the shape and relative position of the curves remain unaffected [174]. The melting profile is based on the length and G/C content of a sequence. Smaller amplicon sizes (up to 400 bp) can increase the sensitivity of detection [174] relative to long amplicons. Given that LAMP amplicons are long concatemers, detection may be variable. As amplicons begin to melt, regions that have higher G/C contents (compared to A/T) are more stable and do not melt immediately [174]. The overall effectiveness of the melt curve analysis is also directly linked to the efficiency of the amplification assay, which can be affected by annealing temperatures of primers to template DNA, copy number of the target genes, and self- annealing of PCR products. SYTO-82 was not used prior to these experiments and as such was not extensively optimized as the calcein assays.

With further development, the parameters of the SYTO-82 assay would be optimised, just as calcein was, and this may result in greater consistency of melt curves across experiments.

SYTO-82 binds to all dsDNA and so has the potential to bind to non-specific amplified products such as primer dimers. Primer dimers are the most common cause of non-specific amplification. The large number of primers targeting regions within a relatively small segment of a genome increases the likelihood of primer dimer interactions [175, 176]. The inner primers are particularly prone to the formation of these structures due to their long length. The concentration of these primers are usually higher than those used in PCR reactions [175]. Other factors include high concentrations of Mg, dNTPs and polymerase [176]. Using melt curve analysis to distinguish between true and non-specific amplification, is beneficial in optimising new assays.

The capability to perform melt curve analysis was only available at the end of this project. Given more time, further work would have included combining this analysis with the T1 workflow. This would determine whether melt curve analysis was still feasible with un-purified extracts.

Using PCR amplicons, melt curve analysis has also been used to distinguish between different species of *Anopheles* vectors [177], and mutations that confer antimalarial [178, 179] and insecticide resistance [180]. This may also be possible with LAMP but was outside the scope of this project.

Malaria is not the only cause of febrile illness in resource limited settings, so a diagnostic panel for other febrile illnesses would also be possible with multiplexed LAMP. A study in Angola reported malaria misdiagnosis in up to 84% of cases [181]; and numerous other studies have shown over-diagnosis of malaria [182-185]. Often it is not practical to perform diagnostic tests for all infections that present with fever related symptoms. As such, providers rely on syndrome-based diagnostic and treatment methods which often result in the prescription of unneeded drugs [182, 185, 186]. These undifferentiated febrile illnesses have been observed with military personnel on deployment in developing countries [187]. Systematically testing every individual for the myriad of infections characterised by acute fever and fever related symptoms (malaria, dengue, chikungunya, Zika, typhoid fever) is not practical in resource-constrained settings. Screening for various pathogens using LAMP

(using melt curve analysis as confirmation) may be an appropriate solution. Such multi-pathogen diagnostic tests have been investigated [188-194], [193] but none utilize LAMP, cover a combination of common non-malaria febrile illnesses, and are suitable for use in low resource settings.

6.5 Conclusion

A simplified sample processing method non-reliant on heating and centrifugation has been developed. This method decreases time to result and is suitable for un-trained users. Melt curve analysis has be used to distinguish between *Plasmodium* species infections and non-specific amplification. This feature shows promise in multiplexing LAMP for other febrile illnesses and for drug and insecticide resistance screening. Future work will involve combining these two findings into one user friendly device, and to improve throughput for greater applicability in elimination settings.

7 General Discussion and Conclusions

To meet the goal of malaria elimination, increased emphasis has been placed on detecting and treating every infection to prevent onward transmission. This requires detection of asymptomatic infections, often amongst migrant and mobile populations [22, 23]; but current diagnostic tests are not sensitive enough to detect the low level parasitaemia. This research has addressed the critical need for field-appropriate, rapid and sensitive malaria diagnosis.

In addressing the need for sensitive malaria diagnostics the research presented in this thesis has: (1) assessed challenges associated with implementing a new technology in resource limited settings; (2) assessed the practicality of LAMP for malaria detection in a field setting using a commercial LAMP kit; (3) made considerable inroads in the development of an in-house LAMP assay that is cost effective and simple to use; and finally (4) investigated a LAMP workflow that is highly suited to use in resource poor settings.

7.1 This thesis in context

Diagnostics play an important role in disease control and elimination, not just in malaria, but in other infectious diseases as well. Their role has been noted in the elimination process in diseases such as hepatitis C [195], measles and rubella [196], human African trypanosomiasis [197] and tuberculosis [198]. Diagnostics are required to detect carriers and to confirm symptomatic cases so that the appropriate treatment can be administered. The role of highly sensitive diagnostics in malaria elimination is important as prevalence declines, as a higher proportion of infected individuals will be asymptomatic [199].

In elimination settings, some suggest that MDA is the most effective strategy to address asymptomatic carriers circumventing the need for diagnosis. However, MDA has shown to be unsuccessful in high transmission settings, large populations, transient populations, and where user adherence is low [12, 200-202]. Treating everyone without first screening has repercussions on the epidemiology of disease transmission. Without incidence and prevalence data it is difficult to ascertain whether an intervention has been successful. Moreover, MDA alone is also not a sustainable solution without other control

measures in place such as effective vector control [12, 203-205]. Community acceptance of MDA should also be considered, as many may be reluctant to take medication in the absence of symptoms for fear of side effects or lack of convenience [206-208]. Given the shortcomings of MDA alone, the role of diagnostics in malaria elimination should not be underestimated. Focused screening and treating may be a more effective regime.

The importance of asymptomatic carriers as reservoirs of infection is established, and thus the need for highly sensitive diagnostic tests is recognised. However, the extent of sensitivity required to prevent transmission is unknown. Gametocytaemia is used as a surrogate marker for infectivity. When using PCR, gametocytes above the threshold of 1 gametocyte/µl of blood were detected in almost half (44%) of the us-RDT negative samples [209]; and other studies have demonstrated the presence of gametocytes in both symptomatic and asymptomatic infections [53, 210]. Elimination efforts therefore cannot solely target asymptomatic trophozoite carriers, but also need to address asymptomatic gametocyte carriers as they appear to also contribute to ongoing transmission.

LAMP has been utilised in gametocyte detection in only one study to date [211]. This assay was capable of detecting <1 gametocyte/µl, enabling it to assess the potential transmission risk that an individual infection poses. Using the melt curve analysis developed in this thesis, it may be possible to develop a multiplex assay that could detect the presence of a *Plasmodium* infection and the level of gametocytaemia. This would inform whether an individual was infected whilst simultaneously assessing their likelihood to transmit; thus informing requirement for treatment. Moreover, a measure of infectivity can guide the need for those in contact with the infected individual to also be tested. Such a concept would need extensive research as a combined multiplexed RT-LAMP and conventional LAMP in a single assay, has not been demonstrated.

While it may seem counterintuitive to rely on instrumentation for a field test, the benefits cannot be overlooked. Using the instruments, minute differences were apparent which made the optimization process very thorough. In a field setting, linking the instrument with the health system database would

allow data to be captured immediately meaning decreased workload for staff and decreased likelihood of errors during manual entering. Instrumentation also removes the onus of decision making from the end user which can be beneficial, primarily as it improves objectivity.

It has been recommended that the Equipment-free criteria in the ASSURED framework be modified to "minimal equipment that can be solar powered"[212] or "no large electricity-dependent instruments needed to perform the test; portable handheld battery-operated devices are acceptable"[213]. The isothermal instruments used in this thesis can be operated standalone connected to a compact rechargeable battery, with a battery life of up to 8 hours per charge. This makes them feasible for field use. The use of GeneXpert for tuberculosis diagnosis (Xpert MTB/RIF) in resource constrained settings has been demonstrated many times with high acceptability from end users [214-217]. The use of this technology requires extensive equipment and constant power supply, much more so than the isothermal instruments described in this thesis. On this basis, LAMP appears to be sufficiently "equipment-free" for application in resource-poor settings.

LAMP has potential beyond malaria diagnosis. Resistance to artemisin in *P. falciparum* is well documented [218-220]. Treatment of *P. vivax* infection with primaquine cannot be initiated treatment unless the G6PD deficiency status of the individual is known [221-223]. Detection of antimalarial resistance and G6PD deficiency may be possible using LAMP technology. Studies have identified potential biomarkers that may indict resistance to artemisinin. These include mutations in the ATPase6 [224, 225], and Pfmdr1 [225, 226] genes. Other studies have developed PCR assays for genotyping G6PD mutations [227-229] and also using melt curve analysis to distinguish between genotypes [228]. These markers have all been successfully amplified by PCR, thereby indicating it may also be possible to detect them with LAMP. Multiplexing LAMP with parasite detection and antimalarial resistance or G6PD deficiency could decrease the burden of the diagnostic workflows in many regions.

7.2 Limitations

Despite the progress made with the in-house assay, it was not as sensitive as PCR. This was resultant from a sudden and unexplainable loss in sensitivity of the in-house assay when this comparison was

made. All efforts to restore the sensitivity previously displayed by the in-house assay were unsuccessful. Thus an accurate comparison of the in-house LAMP assay to PCR was not obtained in this study. Future work will involve re-establishing the sensitivity of the in-house assay and then comparing it again to PCR.

Due to time constraints it was not possible to directly compare the in-house LAMP assay to the Eiken LAMP kit. However, the laboratory data generated in this thesis (**Chapter 5**), where detection of 5 parasites/µl was possible, suggests that the in-house LAMP assay is sufficiently sensitive to trial in a field study, and may compare favourable to the performance of the Eiken LAMP kit (stated to have a LOD of 1 parasite/µl). Comparable sensitivity to PCR is important, but equivalence to the Eiken kit would be the primary objective of a comparison, on the basis that the introduction of a new diagnostic can be justified if it has similar sensitivity and specificity to an existing diagnostic, along with other benefits.

Simplified DNA extraction methods were only explored for whole blood samples, and time constraints meant methods for DBS samples could not be extensively optimised. DBS are a common sampling strategy used in large scale field studies, so the LAMP workflow should be adapted to accommodate these samples.

This study was the most comprehensive study to be conducted on asymptomatic carriage in border areas of Vietnam. However, cross- sectional sampling may not have been the most effective sampling strategy. Although a very large proportion of the community was screened, these individuals may not have been the most at risk members of the community. They did live in border areas, but it was not known whether they engaged in forest activities, travelled across borders, or were migrants. Perhaps a more informative approach would have been to identify at risk index cases (individuals that work or sleep in the forest, or travel regularly across borders) and use active case detection to sample individuals around them. This would have enabled a clearer understanding of asymptomatic carriage in these communities.

The qualitative data and operator feedback obtained during the LAMP implementation was insightful. More could be learned if the study incorporated more avenues for qualitative data collection. In particular, obtaining feedback from more LAMP operators would have been useful. Feedback from Commune and Village Health Care Workers would have been valuable as they were the intended end users. Obtaining responses that were more quantifiable (i.e. on a scale of one to ten) rather than verbal responses would also be preferred. Feedback would then be easier to analyse and would be less open to interpretation by study organisers. Feedback from participants would have be useful to gain an insight into community acceptance.

7.3 Translational implications

Implementation of any new technology in resource limited settings is likely to face a unique set of challenges. However, not all challenges are new or entirely unique, and it is likely that future diagnostic implementation studies will face some challenges similar to those faced in this study. As such, the observations made in the implementation of LAMP in Vietnam as described in **Chapter 3** may be useful for researchers planning an implementation field study. There is potential for this study to inform training, study design and rollout of implementation.

The LAMP assay developed in this body of work can be adapted to any pathogen. The optimization process would be valuable when developing a new in-house assay regardless of the target infection. The optimization with calcein and SYTO as detection dyes, as well as their integration with the isothermal instruments for real time detection, can be applied to any LAMP reaction. The T1 sample processing workflow should be compatible with other assays using whole blood, and potentially other assays with liquid samples (urine, saliva). This would enable simple and cost affordable diagnostic tests for many diseases.

7.4 Future directions

The detection modality used for optimisation of the in-house assay was calcein and the melt curve analysis was performed using SYTO. Further optimization should be carried out with SYTO to ensure that it is also compatible with the simplified sample processing workflow. This would enable sensitive detection with minimally processed samples, whilst also having the ability to multiplex the assay in one cartridge.

To further develop the in-house assay, capabilities to multiplex parasite detection with transmission potential, drug resistance, or G6PD deficiency should be explored. This may involve re-optimizing the assay to account for additional primers and specific template requirements (e.g. introduction of betaine or BSA if template DNA/RNA is GC rich or prone to forming secondary structures).

Future work to improve the stability of the LAMP reagents is also critical. This would involve optimisation of the lyophilisation process and may involve investigating alternative reagents if they are not compatible (the glycerol in *Bst* may impact its ability to dry completely). Once dried, the stability of the reagents should be tested under various temperatures and humidity to determine their shelf life and their field applicability.

Once the appropriate modifications have been made the in-house assay, it should be trialled in a resource-limited field setting. For a direct comparison, trialling the assay in the same sites as the RAI study would be ideal. The study could be collaboratively designed with health care staff in Vietnam to ensure acceptability. Amendments to the study design may include investigating more direct data entry either into one of the existing databases or by using the isothermal instruments to upload data directly. And finally, an active case screening approach should be taken to sampling, to ensure that prevalence in the most at risk members of the community is captured.

7.5 Conclusion

Highly sensitive diagnostic tests play a critical role in malaria elimination. This thesis provided an insight into the utility of LAMP as a screening tool for asymptomatic infections in a resource

constrained setting. Using a commercial LAMP assay to screen the largest population in this region to date, a previously unreported asymptomatic reservoir was uncovered in South East Vietnam.

Analysis of the processes involved in implementing this tool, uncovered an array of important but often overlooked external factors to consider when conducting a large scale field trial. These findings can be applied to any study design. Findings from this study emphasised the importance of acceptability among end users and policy makers, comprehensive and relevant training of field staff, and managing logistical demands.

An in-house LAMP assay was developed to address the unmet need of affordable molecular testing. The assay was combined with instrument detection to allow for faster time to results and multiplexed detection. Combining the in-house assay with simplified sample preparation, further increased its applicability in field settings without compromising sensitivity.

Future work will involve increasing the stability of the in-house LAMP assay so that it may be more easily implemented in field settings, and to trial the assay in a low resource setting to determine its sensitivity, ease of use, and acceptability from end users.

Appendix 1: Primer and probe sequences

Sequences of primers and probes used in LAMP and PCR assays. 18s rRNA in-house LAMP primers were published in [70], mDNA in-house LAMP primers were published in [71], and 18s rRNA PCR primers and probes were provided by Ms Franca Azzato (VIDRL).

In-house LAMP assay

18s rRNA target

Species	Primer	Sequence
Plasmodium genus	FIP	5'- TCGAACTCTAATTCCCCGTTACCTATCAGCTTTTGATGTTAGGGT-3'
	BIP	5'- CGGAGAGGGAGCCTGAGAAATAGAATTGGGTAATTTACGCG-3'
	F3	5'- GTATCAATCGAGTTTCTGACC-3'
	B3	5'- CTTGTCACTACCTCTCTTCT-3'
	LF	5'- CGTCATAGCCATGTTAGGCC-3'
	LB	5'- AGCTACCACATCTAAGGAAGGCAG-3'
P. falciparum	FIP	5'- AGCTGGAATTACCGCGGCTG GGTTCCTAGAGAAACAATTGG-3'
	BIP	5'- TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATGAAAC-3'
	F3	5'- TGTAATTGGAATGATAGGAATTTA-3'
	B3	5'- GAAAACCTTATTTTGAACAAAGC-3'
	LF	5'- GCACCAGACTTGCCCT-3'
	LB	5'- TTGAATATTAAAGAA-3'
P. vivax	FIP	5'- CTATTGGAGCTGGAATTACCGCTCCCAAAACTCAATTGGAGG-3'
	BIP	5'- AATTGTTGCAGTTAAAACGCTCGTAAGCTAGAAGCGTTGCT3-'
	F3	5'- GGAATGATGGGAATTTAAAACCT-3'
	B3	5'- ACGAAGTATCAGTTATGTGGAT-3'
	LF	5'- GCTGCTGGCACCAGACTT-3'
	LB	5'- AGTTGAATTTCAAAGAATCG3'

mDNA target

Species	Primer	Sequence
P. falciparum	FIP	5'- ACCCAGTATATTGATATTGCGTGACAGCCTTGCAATAAATAATATCTAGC-3'
	BIP	5'- AACTCCAGGCGTTAACCTGTAATGATCTTTACGTTAAGGGC-3'
	F3	5'- CTCCATGTCGTCTCATCGC-3'
	B3	5'- AACATTTTTTAGTCCCATGCTAA-3'
	LF	5'- CGGTGTGTACAAGGCAACAA-3'
	LB	5'- GTTGAGATGGAAACAGCCGG-3'

PCR assay

18s rRNA target

Species	Primer/probe	Sequence
Plasmodium genus	Forward	5'- ACATGGCTATGACGGGTAAC- 3'
	Reverse	5'-ÇTTCCTTAGATGTGGTAGCTATTTCTCA-3'
	Probe	FAM6-AATTAGAGTTCGATTCCGGAGAG-MGBNFQ
P. falciparum	Forward	5'-TTTTGGCTTTAATACGCTTCCTCTA-3'
	Reverse	5'- AACAAAATCCCCAAAAAAGCAA-3'
	Probe	FAM6-TTTTAAAATCCCCACTTTTG-MGBFQ
P. vivax	Forward	5'-AGCTTAATCCACATAACTGATACTTCGT -3'
	Reverse	5'-CCGAAGCAAAGAAAGTCCTTAAAA-3'
	Probe	FAM6-ACTTTGTGCGCATTTT-MGBNFQ

Appendix 2: Standard Operating Procedures developed for RAI study

The following are all the SOPs developed for the RAI study. SOPs were developed in collaboration with NIMPE and FIND. SOPs were used in field training and were available to staff throughout the study for reference.

SOP 1: LAMP preparation

SOP1: Protocol for LAMP preparation

Effective from:	TBA
Revision history:	**Authors:** – **Burnet Institute** (Leanna Surrao, Dr Vashti Irani, Duy Thanh Vo, Dr Jack Richards) – **Foundation for Innovative New Diagnostics** **Date:** 03/09/2017 **Translated:** Nguyen Xuan Thang, Duy Thanh Vo **Date:** 03/09/17 **Reviewed by:** **Date:** **Next review due:** **Signature(s):**

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Abbreviations
SDS: Sodium dodecyl sulfate
NaCl: Sodium chloride
SOP: Standard operating procedure

Part 1-Preparation of extraction tubes

Purpose
This SOP describes how to prepare the DNA extraction tubes for LAMP testing. This buffer needs to be prepared in an established laboratory beforehand as such preparation will be outside the scope and resources of a village health centre. This SOP is to be used for preparing for the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study.

Reagents
- Tris base (#TB0196)
- Hydrochloric acid
- Sodium dodecyl sulfate (SDS) (#SB0485)
- NaCl (#7647-14-5)
- Molecular grade water

Equipment
- Mechanical pipette 100-1000µl (#3120000062) with disposable pipette tips (#23-0200)
- Disposable non-sterile examination gloves (#VGP-6602)
- 1.5ml tubes (#11510)
- Rack for 1.5ml tubes
- 100ml and 10ml measuring cylinders
- 100ml Schott bottle, or similar container (2x)
- pH meter
- Biological hazard waste bin
- Permanent marker

- Weighing scale

Standard operating procedure

General guidelines

- Conduct the following steps in an established laboratory prior to commencing field testing
- Ensure environment is clean and the reagents and consumables are kept sterile when possible
- This protocol makes 100ml of extraction buffer. This is enough for approximately 1,666 extraction tubes. Amounts can be scaled depending on how many extraction tubes are required
- Each component must be made separately and then combined in the appropriate ratios
- Each component of the extraction buffer is necessary to extract parasite DNA from the red blood cell

Step-by-step actions

1. **PREPARE** 100mM **Tris HCl** solution by adding **484.56mg of Tris** to **40ml of water**. Once dissolved, check the pH of this solution using the pH meter. Adjust the pH to 6.4 by slowly adding HCl drop by drop. Set this solution aside
2. **PREPARE** 10% **SDS** solution by adding **10g of SDS** to **80ml of water**. Once dissolved, **top up to 100ml** with more water. The SDS may take some time to dissolve- if so, heating up in the mixture in a water bath, incubator, or warm area of the lab can help. Set this solution aside
3. **PREPARE** 5M **NaCl** solution by adding **2.3g of NaCl** to **8ml of water.** Set this solution aside
4. **PREPARE** 100ml Schott bottle (or similar container) by labelling it with "extraction buffer" and the date of preparation
5. **PREPARE** the extraction buffer by adding **48ml of molecular grade water**, **40ml of the 100mM Tris HCl solution**, **4ml of the SDS solution**, and **8ml of the NaCl solution**
6. **ALIQUOT 60µl of the extraction buffer** into individual 1.5ml tubes using the mechanical pipette. Change the pipette tip after every 10 tubes aliquoted to limit the chance of incorrect pipetting
7. One tube is required for each patient so 11,000 tubes should be aliquot (10,000 patients will be sampled in the cross sectional survey and 1000 should be made as extras in case they are needed)
8. **LABEL** all tubes with red dot sticker and store at room temperature away from direct sunlight

Part 2- Preparation of dilution tubes

Purpose

This SOP describes how to prepare the DNA dilution tubes for LAMP testing. This needs to be prepared in an established laboratory beforehand as such preparation will be outside the scope and resources of a village health centre. This SOP is to be used for preparing for the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study.

Reagents

- Molecular grade water

Equipment

- Mechanical pipette 100-1000µl (#3120000054) with disposable pipette tips (#23-0200)
- Disposable non-sterile examination gloves (#VGP-6602)
- 1.5ml tubes (#11510)
- Rack for 1.5ml tubes
- Biological hazard waste bin

Standard operating procedure

General guidelines

- Conduct the following steps in an established laboratory prior to commencing field testing
- Ensure environment is clean and the reagents and consumables are kept sterile when possible
- The dilution buffer is necessary to dilute out inhibitors that will be in the extract. It is important to remove these biological inhibitors otherwise they will prevent the LAMP reaction from working effectively

Step by step actions

1. **ALIQUOT 345 µl of molecular grade water** into individual 1.5ml tubes using the mechanical pipette. Change the pipette tip after every 10 tubes aliquoted to limit the chance of incorrect pipetting
2. One tube is required for each patient so 11,000 tubes should be aliquot (10,000 patients will be sampled and 1000 should be made as extras in case they are needed)

3. **LABEL** all tubes with yellow dot sticker and store at room temperature away from direct sunlight

END OF SOP FOR EXTRACTION AND DILUTION TUBE PREPARATION

Part 3- Preparation of sample collection kits

Purpose

This SOP describes how to prepare sample collection kits before commencing samples collection in the field. This needs to be prepared as the logistical requirements to prepare them will be outside the scope and resources of a village health centre. This SOP is to be used for preparing for sample collection for the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study.

Material and Equipment

- Plastic zip lock bags
- Barcode sets
- Marker
- Scissors

Standard operating procedure

Step-by-step actions

1. Unique barcodes for each sample have been printed on large rolls
2. Each sample will have **2 large barcodes** and **10 small barcodes** with the same number
3. **CUT** each set of barcodes with a scissors
4. **PLACE** a set of barcodes in a small plastic zip lock bag ensuring the numbers on all 12 barcodes match
5. **STORE** at room temperature away from direct sunlight until samples are ready to be collected

END OF SOP FOR PREPARING SAMPLE COLLECTION KITS

SOP 2: Site setup

SOP2: Protocol for LAMP set up at new site

Effective from:	TBA
Revision history:	**Authors:** – **Burnet Institute** (Leanna Surrao, Dr Vashti Irani, Duy Thanh Vo, Dr Jack Richards) – **Foundation for Innovative New Diagnostics** **Date:** 03/09/2017 **Translated:** Nguyen Xuan Thang, Duy Thanh Vo **Date:** 03/09/17 **Reviewed by:** **Date:** **Next review due:** **Signature(s):**

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Abbreviations

SOP: standard operating procedure
DBS: dried blood spot
RDT: rapid diagnostic test
UV: ultraviolet

Purpose

This SOP describes how to set up a new testing site including all the equipment and materials required. As LAMP testing has not been conducted in commune health care centres until this study each site will need to be set up prior to any testing. This SOP is to be used in the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study.

Reagents

- Tub or bag of extraction tubes (red lids)
- Tub or bag of dilution tubes (yellow lids)
- Rapid diagnostic tests (RDTs) (#05FK83)
- Pan LAMP kits (#LMC562)

Equipment

- Mechanical pipettes 2-20ul (#3120000038) and 20-200ul (#3120000054) with disposable pipette tips (#23-0200)
- Microscope slides
- Filter paper strips for DBS (#3030-917)
- Zip lock bags
- Desiccant pouches
- Heat blocks (2x) (#DBH10D)
- Heat block adapters (1x 1.5ml tubes size #EB20)and 2x LAMP #EB48 tubes size)
- Benchtop centrifuge (#MPW55)
- Mini centrifuge (#6766-230V)
- UV light box (#DL-107)
- UV light (#DL-01) + batteries
- UV safety glasses
- All relevant SOPs

- Waste bin/bags
- 1.5ml tube racks
- LAMP tube racks
- Permanent markers
- Pens
- Power point adapter/power board

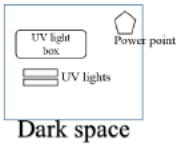
Standard operating procedure

General guidelines

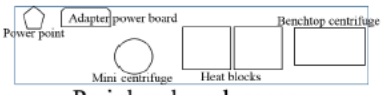
- Ensure the work site is set up prior to any samples testing
- Ensure the work space is set up in a way that reflects the work flow to ensure simplicity and efficiency

Step-by-step actions

1. **SETUP** the tubs/bags of extraction tubes and dilution tubes, RDTs, microscope slides, filter paper, desiccant pouches, zip lock bags, Pan LAMP kits, on one side of the immediate work space
2. **SETUP** the mechanical pipettes and disposable tips, all relevant SOPs and documentation, 1.5ml tube and LAMP tube racks, waste bins/bags, pens, and markers one the other side of the immediate work space
3. **SETUP** the centrifuges and heat blocks next to each other in the peripheral work space near a power point. Make sure they are turned on and ready to use. The peripheral work space may be the same as the immediate workspace given that power points are accessible
4. **SETUP** the UV lights and UV box in the dark space (dark part of the room). Ensure UV lights have batteries and are working. Ensure the UV light box is plugged in and ready to use. If there are no power points accessible in the dark space, then the UV light box may be moved to the peripheral work space. *NOTE: dark conditions will be necessary to view UV results. This can be achieved by turning off lights or interpreting results in a dark cupboard or draw*
5. **LABEL** the 1.5ml racks with labelling tape. One will be "Rack A", the next "Rack B", the next "Rack C", and the last "Rack D".

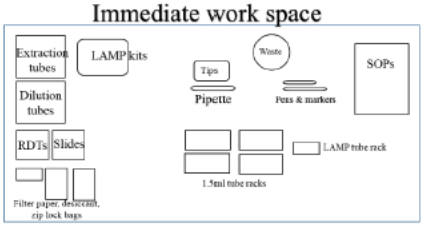


Dark space



Peripheral work space

Immediate work space



END OF SOP FOR SETTING UP LAMP IN NEW SITE

SOP 3: Sample collection

SOP3: Protocol for sample collection and labelling

Effective from:	TBA
Revision history:	**Authors:** – **Burnet Institute** (Leanna Surrao, Dr Vashti Irani, Duy Thanh Vo, Dr Jack Richards) – **Foundation for Innovative New Diagnostics** **Date:** 03/09/2017 **Translated:** Nguyen Xuan Thang, Duy Thanh Vo **Date:** 03/09/17 **Reviewed by:** **Date:** **Next review due:** **Signature(s):**

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Abbreviations:

SOP: Standard operating procedure

Purpose:

This standard operating procedure (SOP) describes how to collect blood samples from participant's in rural villages prior to further testing at the commune health centre. This SOP is to be used for blood collection of patients in the cross-sectional survey in the Malaria Control and Outreach for MMPs Along the Vietnam-Cambodia border study.

Reagents and Equipment

- Sample collection kit containing 1 set of unique barcodes (1 per participant) prepared in SOP 1
- Heparin microtainer (1 per participant) (#365965)
- Disposable alcohol wipes (1 per participant) (#13485 2003)
- Disposable sterile lancets (1 per participant) (#63300)
- Microscope slides (1 per participant)
- Disposable non-sterile examination gloves (1 pair per participant) (#VGP-6602)
- Hand wash basin with soap or alcohol hand rub
- Cross sectional patient form (1 per participant)
- Consent form (1 per participant)
- Pens
- Rubbish bags
- Band-aids
- Large zip lock bags

Potential hazards and safety considerations

There is a possible risk of infection when collecting and working with blood samples and of working with sharps such as lancets. Ensure personal protective equipment and caution is taken to minimize said risk.

Standard operating procedure

General guidelines

- Ideal prick sites are the tips of the 3rd or 4th finger (middle finger or ring finger)

Step-by-step actions

1. **EXPLAIN** the study to the participant and inform them of how their sample will be used
2. If they agree to participate, ask them to **SIGN** the consent form
3. **COMPLETE** a cross sectional patient form. Refer to *SOP 6* for detailed protocol
4. **COLLECT** a sample collection kit
5. **ENSURE** all the numbers on the barcodes are the same. If they are not, discard that sample collection kit and use one where all barcodes have the same number
6. **ATTACH** a large barcode on the outside of the sample collection kit
7. **ATTACH** a large barcode on the patient's form in the space provided (bottom of first page)
8. **DISCARD** the backing for the large barcodes in the rubbish bag
9. **ATTACH** a small barcode on a heparin microtainer. Ensure the label is placed length ways so that so that the barcode can be read properly
10. **ATTACH** a small barcode to the microscope slide
11. **WASH** your hands carefully using the available wash basin and soap or using an alcohol hand rub.
12. **PUT ON** adequately sized examination gloves.

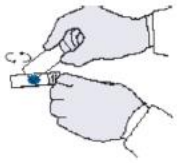
    Always wear gloves and change them if you get blood on them
13. **DESINFECT** the prick site by rubbing it with an alcohol wipe and allowing it to air dry.

14. **DISCARD** of this alcohol wipe in the rubbish bag provided
15. **PRICK** the disinfected site with a disposable sterile lancet and **IMMEDIATELY** put the lancet back in its paper bag and discard in the rubbish bag provided

16. **COLLECT** the first drop of blood onto the microscope slide. Place it in the middle of the slide and set it aside
17. **COLLECT** blood in microtainer by dripping blood directly from finger prick into the microtainer provided. Rest the finger on the edge of the tube and allow the blood to flow into the tube by capillary action. It might be necessary to rub the finger gently in a downwards direction to stimulate blood flow and allow more blood to be collected in the tube. If bleed is insufficient and the level has not reached the first line on the microtainer (200µl), repeat on another finger until at least 200ul of blood is collected.

18. **CLOSE** microtainer and place back in the corresponding sample collection kit

    Make sure blood is always contained and samples never mix
19. **PLACE** band-aid on participant's finger to stop the bleeding
20. Use the edge of another slide to spread the sample. Use a **CIRCULAR MOTION** to spread the sample into a small 1-2mm dot.



    Don't make the smear to thick or it will fall off the slide when staining. It should be thin enough that you can see through it
21. **ALLOW** slide to dry completely before storing
22. **ONCE** dried place in corresponding sample collection kit

23. **MARK** the sample collection kit with a "M" to signify that a microscopy slide has been made for this sample
24. **SEAL** the sample collection kit and fold form around kit (fold form in half and place and sample collection kit inside the folder form)
25. **PLACE** patient form and sample collection kit inside large zip lock bag
26. **CONTINUE** to collect patient samples following the above steps and transport to commune health centre for LAMP processing

END SOP FOR SAMPLE COLLECTION

SOP 4: LAMP testing

SOP4: Protocol for DNA extraction and LAMP testing

Effective from:	TBA
Revision history:	**Authors:** – **Foundation for Innovative New Diagnostics** – **Burnet Institute** (Leanna Surrao, Dr Vashti Irani, Duy Thanh Vo, Dr Jack Richards) **Date:** 03/09/2017 **Translated:** Nguyen Xuan Thang, Duy Thanh Vo **Date:** 03/09/17 **Reviewed by:** **Date:** **Next review due:** **Signature(s):**

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Abbreviations:

SOP: Standard operating procedure

UV: Ultra-violet

DNA: Deoxyribonucleic acid

LAMP: Loop-mediated isothermal amplification

HCl: hydrochloric acid

SDS: sodium dodecyl sulfate

NaCl: sodium chloride

Purpose:

This SOP describes how to extract DNA from patient samples and how to perform and interpret LAMP reactions. This SOP is to be used for blood samples collection from patients in the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study.

Reagents:

- Loopamp™ MALARIA Pan detection kit (#LMC562)
  - Malaria Pan reaction tubes with detection reagents
  - Pan Positive Control (PC)
  - Pan Negative Control (NC)
- Sample collection kits (1 per participant)
- 0.5% sodium hypochlorite/ bleach (in spray bottle)
- Extraction control
- Extraction (contains extraction buffer made of Tris-HCl, SDS, and NaCl) tubes (1 per participant)
- Dilution tubes (contains water) (1 per participant)

Equipment:

- 2x heat block (#DBH10D)
- 1x heat block adapter for 1.5 mL tubes (#EB20)

- 2x heat block adapter for LAMP tubes (#EB48)
- LAMP reaction record sheet
- Cross sectional patient forms (1 per participant)
- 20-µl to 200-µl micropipette (#3120000038) with sterile disposable tips (#23-0200)
- Disposable non-sterile examination gloves (#VGP-6602)
- 4x Rack for 1.5ml tubes
- 1x Rack for LAMP reaction tubes
- Permanent markers
- Pens
- Benchtop centrifuge (#MPW55)
- UV light (#DL01)+ batteries (4x AA)
- UV light box (#DL-107)
- Timer
- Waste bin/bag
- Labelling tape
- Mini centrifuge (#6766-230V)
- Tissues

Potential hazards and safety considerations

There is a possible risk of infection when working with blood samples and chemicals such as the extraction buffer and bleach. Ensure personal protective equipment and caution is taken to minimize said risk. Used LAMP tubes also present a great contamination risk. NEVER open used LAMP tubes and also dispose of safely.

General guidelines

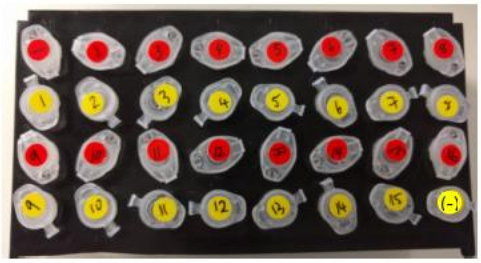
- The heating block for 1.5 mL tubes (DNA extraction) can accommodate up to 20 tubes. It is recommended to process only **16 DNA EXTRACTIONS (15 SAMPLES AND 1 CONTROL) SIMULTANEOUSLY**
- The heating block for LAMP tubes (LAMP reaction) can accommodate up to 48 tubes. It is recommended to process only **32 (30 SAMPLES + 2 CONTROLS) LAMP REACTIONS SIMULTANEOUSLY**
- **NEVER** open LAMP reaction tubes after the reaction to avoid contamination.

Step-by-step actions

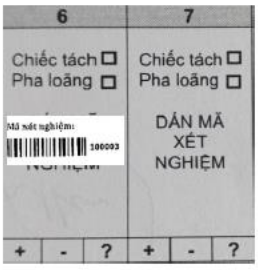
1. **INSTALL** the 1.5 mL adapter into the heating block, turn it on, and **SET THE TEMPERATURE AT 95 °C**
2. **INSTALL** the LAMP tube adapter into the heating block, turn it on, and **SET THE TEMPERATURE AT 65 °C**
3. **CLEAN** the work area (bench, racks, and pipette) with 0.5% sodium hypochlorite by spraying with spray bottle and wiping with tissues
4. **PLACE** on the bench all required materials for 2 sets of DNA extraction:

a. **x30** sample collection kits with corresponding cross- sectional patient forms
b. **x32** extraction tubes (with a red sticker on the lid)
c. **x32** dilution tubes (with a yellow sticker on the lid)
d. **x1** 20 µL to 200 µL micropipette with sterile disposable tips
e. **x4** 1.5ml tube racks
f. **x1** LAMP tube rack

5. **COMPLETE** the LAMP reaction sheet. The run ID should be in the following format; "date(dd/mm/yy)/time(24 hour time)". For example a run that was performed on the 3rd of September at 9 am will have the run ID of "030917/0900"
6. **LABEL** each of the four 1.5ml tube racks. The first rack will be called "Rack A", the next "Rack B", the next "Rack C", and the final rack "Rack D"
7. **PLACE** 16 extraction and dilution tubes on Rack A. **LABEL** the lids of each extraction and dilution tube with numbers from 1-15. LABEL the 16th tube with a negative sign (-) as this will be the extraction control



8. **REMOVE** the microtainer and barcodes from a sample kit
9. **PLACE** one small barcodes on the extraction tube, and one small barcode on the LAMP reaction record sheet. Ensure labels on tubes are placed lengthwise so the barcode can be scanned properly



10. **TRANSFER 60µl** of blood from the microtainer to the extraction tube labelled 1. *Always wear gloves and change them if you get blood on them*
11. **MIX** the extraction tubes by pipetting up and down 5 times
12. **DISPOSE** of tip in biohazard bin
13. **RETURN** the sample back to its corresponding sample collection kit and label the bag with an "L" to signify that LAMP has been run for this sample
14. Once mixed, **TRANSFER** extraction tube 1 to Rack B to signify that this sample has been processed

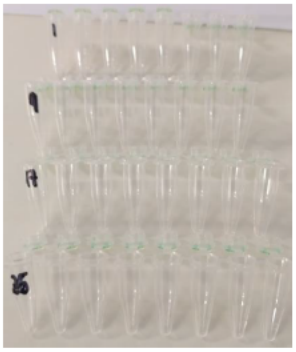
15. On the LAMP reaction sheet, **TICK THE BOX** that says "extraction" to signify that this step in the protocol has been completed
16. **REPEAT** steps 8-14 for the remaining 14 samples starting at tube 2, then tube 3, and so on
17. **INCUBATE** the samples at **95 °C** for **5 minutes** in the pre-heated heating block.

    DO NOT INCUBATE MORE THAN 5 MINUTES. *Ensure the lids of tubes are closed firmly before heating*

Problem: I am ready to incubate my samples but the heat block is not up to temperature yet **Solution:** Ensure the heat block is set to the right temperature and wait until it heats up. It is ok for your samples to sit in extraction buffer for a few extra minutes

18. **REMOVE** the tubes immediately and **CENTRIFUGE** them at room temperature for **3 MINUTES** at **10'000 G**. *Ensure centrifuged is balanced before starting. Ensure the lids of tubes are closed firmly before spinning*

19. **REMOVE** these tubes from the centrifuge and place on Rack A (with the corresponding dilution tubes)
20. **SET** aside Rack A
21. **PLACE** 16 extraction and dilution tubes on Rack C. **LABEL** the lids of each extraction and dilution tube with numbers from 16-30. **LABEL** the 31st tube with a negative sign (-) as this will be the extraction control
22. **REPEAT** steps 8-18 for samples 16-30 and the extraction control for this extraction batch
23. **SET UP** 4 rows of LAMP tubes on the LAMP rack and label the start of each row to keep track of the orientation of the samples. The first strip will be labelled with a 1, the second a 9, the third a 17, and the fourth a 25



24. **TRANSFER 30 µL** of supernatant from tube 1 extraction to the corresponding dilution tube

    The precipitated haemoglobin makes a brown-red pellet at the bottom of the tube. Make sure that none of the precipitate is taken with the tip of the pipette

Problem: Can't access supernatant after centrifugation **Solution:** Spin again for 3 minutes at 10,000 xg and try again

Problem: Not enough DNA extract (less than 30ul) **Solution:** Just use whatever extract there is and make a note on the LAMP reaction sheet. Write "<60ul" above the barcode for this sample on the sheet

Problem: The DNA extract is more pigmented than usual **Solution:** It can still be used but make a note on the LAMP reaction sheet-write "pigmented extract" above the barcode for this sample on the sheet. This is because the pigmentation may affect the colour of the LAMP reaction slightly and might make the result harder to interpret

25. **MIX** the dilution tube by pipetting up and down 5 times Using the same tip, **TRANSFER 30 µL** from the dilution tube to the first LAMP tube of the first strip. Close the lid of the LAMP tube to signify that the LAMP tube has been prepared

Problem: I did not dilute the extract and instead put it straight into the LAMP assay accidentally
Solution: Discard the LAMP tube that the undiluted extract was placed in and take another LAMP tube. Take another 30ul from the same extraction tube, mix it into the dilution tube, and then add to LAMP tube. If there is not enough extract to do take another 30ul, the original 30ul may be withdrawn from the LAMP tube and then diluted properly before being placed into a new LAMP tube

26. **DISPOSE** of tip in waste bin/bag
27. On the form, **TICK THE BOX** that says "dilution" to signify that this step in the protocol has been completed
28. **REPEAT** steps 23-27 for all remaining samples starting with tube 2, then tube 3, and so on

    DO NOT discard extraction and dilution tubes as they may be needed if the run fails and the batch needs to be repeated
29. Using the mini centrifuge and the 1.5ml adapter, **SPIN** the positive and negative controls from the kit to collect their contents at the bottom of the tubes.
30. **PREPARE** the control tubes by removing them from the pack and labelling them with "+" for the positive control and a "-"for the negative control. They will be already split into strips of 2

31. **MIX** the negative control by pipetting up and down 5 times then **TRANSFER 30 µL** of solution into the negative control LAMP reaction tube and close the cap immediately.
32. **DISPOSE** of tip in waste bag/bin
33. **MIX** the positive control by pipetting up and down 5 times then **TRANSFER 30 µL** of solution into the positive control LAMP reaction tube and close the cap immediately.

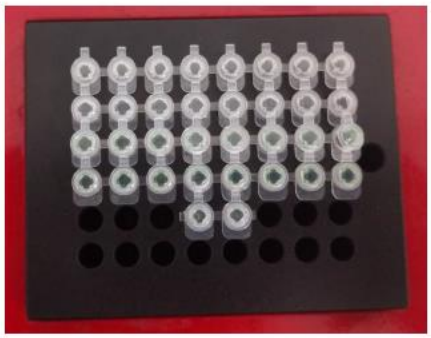
    ALWAYS PREPARE THE POSITIVE CONTROL LAST TO AVOID CONTAMINATION.
34. **DISPOSE** of tip in waste bag/bin
35. **TURN ALL THE LAMP TUBES UPSIDE DOWN AND FLICK FIRMLY** to collect the DNA solution in the tube caps.

36. **INCUBATE FOR 2 MINUTES** at room temperature (the LAMP reagents are dried down in the tube cap and this step is necessary to dissolve them).
37. **INVERT** and **FLICK FIRMLY** the LAMP tubes 10 times to mix and dissolve the LAMP reagents (completing 5 up and down cycles).

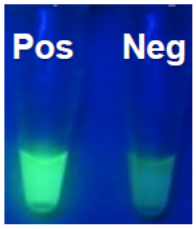
38. Finally, **SPIN** the tubes in the mini centrifuge to collect the solution at the bottom of the tubes.

    Ensure all the solution is at the bottom of the tubes and there aren't many bubbles. If there are bubbles, flick the sides of the tubes gently until they disappear

39. **PLACE** the LAMP tubes in the pre-heated heating block and **INCUBATE AT 65 °C FOR 40 MINUTES.**



40. During incubation, **INSERT** the other LAMP tube adapter into the **95°C** heat block that was used for the DNA extraction step and change the temperature to **80 °C** in preparation for the termination step. Use tissues to hold the 1.5ml tube adapter as it will be very hot
41. During incubation perform other testing according to *SOP5*
42. After **40 minutes**, **REMOVE** LAMP tubes from the **65 °C** heat block and place in the **80 °C** heat block
43. **INCUBATE** the tubes at **80°C FOR 5 MINUTES** to stop the reaction
44. **DETECT** and **INTERPRET** the results.
45. Place the tubes in the UV light box and observe the coloration of the tubes:
    a. If green light is emitted: DNA has been amplified and the reaction is positive.
    b. If no light is emitted: no DNA has been amplified and the reaction is negative.



46. Observe **THE LAMP POSITIVE AND NEGATIVE CONTROLS AND THE EXTRACTION NEGATIVE CONTROLS** first:
    a. If the positive control is positive and the negative controls are negative the run is **VALID** and sample reactions can be interpreted as positive or negative.
    b. If the controls are not as expected, the run is **INVALID** and the sample reactions should not be interpreted. In case of invalid results, LAMP reactions for all samples in the run should be repeated. If this is the case- repeat steps 8-45.
47. **REPORT** the run results on the reaction sheet by **CIRCLING THE RESULT** of the tube

Problem: Unsure if sample is positive or negative **Solution:** If the sample doesn't look exactly like either the negative or positive control, mark it as "unsure (?)" and repeat DNA extraction and LAMP for this sample

48. **RECORD** all of these results on the cross sectional patient form

49. **SEAL** the used LAMP reaction tubes in a ziplock bag, and dispose in waste bin/bag

    NEVER OPEN USED LAMP REACTION TUBES after the reaction to avoid contamination.

50. **COLLECT** the microtainer samples from this batch and place in medium zip lock bag. Record the run ID number on the bag using a marker. Store this bag in the fridge until further use

51. If run was valid, **DISPOSE** of extraction and dilution tubes in waste bag/bin. If it was not valid, **KEEP** extraction tube until the batch is re-run. **DISCARD** the dilution tube

END OF SOP FOR DNA EXTRACTION AND LAMP REACTION

SOP 5: RDTs, microscopy, and DBS preparation

SOP5: Protocol for rapid diagnostic test, microscopy, and dried blood spot preparation and testing

Effective from:	TBA
Revision history:	**Authors:** – **Burnet Institute** (Leanna Surrao, Dr Vashti Irani, Duy Thanh Vo, Dr Jack Richards) – **Foundation for Innovative New Diagnostics** **Date:** 03/09/2017 **Translated:** Nguyen Xuan Thang, Duy Thanh Vo **Date:** 03/09/17 **Reviewed by:** **Date:** **Next review due:** **Signature(s):**

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	Dr Vashti Irani Email: vashti.irani@burnet.edu.au Ph: +61 42325 3306
	Dr Jack Richards Email: jack.richards@burnet.edu.au Ph: +61 3 9282 211

Abbreviations
SOP: Standard operating procedure
RDT: Rapid diagnostic test
DBS: Dried blood spots

Part 1

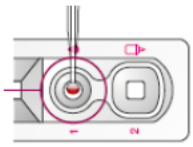
Purpose
This SOP describes how to prepare and run an RDT from a pre-collected patient sample. This SOP is to be used for sample processing of patients in the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study. These tests can be performed during the 40 minute LAMP incubation step.

Material and Equipment
- 2-20µl mechanical pipette (#3120000038) with disposable pipette tips (#23-0200)
- Sample collection kit
- 1 RDT (per patient) (#05FK83)
- Marking pen
- Waste bin/bag

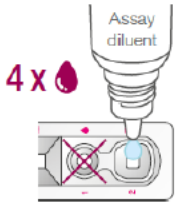
Standard operating procedure
Step-by-step actions

1. **COLLECT** a sample collection kit
2. **PREPARE** an RDT by attaching a barcode onto it
3. **ADD** 6µl of blood to the round hole of the RDT

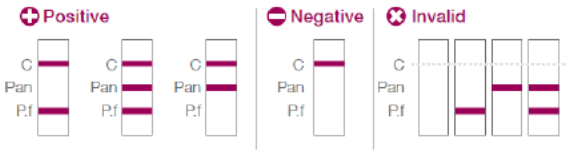
> **Problem:** There is not enough sample to do the LAMP, RDT, and DBS
> **Solution:** When there is not enough sample the LAMP should take priority, followed by the DBS, and then the RDT



4. Add 4 drops of the assay diluent to the square hole of the RDT



5. **RECORD** the time by writing it on the RDT with a pen or marker
6. **MARK** the sample collection kit with a "R" to signify that an RDT has been processed for this sample
7. Wait 15 minutes to interpret the results
8. If the control line is present, the assay is valid and the result can be interpreted. If it is not present, the assay is not valid and must be discarded and repeated with another RDT



9. **Record** this result on the cross sectional patient form
10. **DISCARD** RDT after result has been observed

Part 2

Purpose

This SOP describes how to prepare thick and thin film microscopy slides from a pre-collected patient sample. This SOP is to be used for sample processing of patients in the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study. These tests can be performed during the 40 minute LAMP incubation step.

Material and Equipment

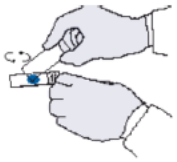
- 2-20µl mechanical pipette (#3120000038) with disposable pipette tips (#23-0200)
- Sample collection kit
- Microscope slides (1 per patient)
- Marking pen

Standard operating procedure

NOTE: if a microscopy slide was not prepared during SOP3, one needs to be made now

Step-by-step actions

1. **COLLECT** a sample collection kit
2. **PREPARE** a microscope slide by attaching a barcode onto it
3. **ADD** 6 µl of sample to the middle of a slide
4. Use the edge of another slide to spread the sample. Use a **CIRCULAR MOTION** to spread the sample into a small 1-2mm dot.



5. Don't make the smear to thick or it will fall off the slide when staining. It should be thin enough that you can see through it
6. **ALLOW** slide to dry completely before storing
7. **ONCE** dried place in corresponding sample collection kit
8. **MARK** the sample collection kit with a "M" to signify that a microscopy slide has been made for this sample

Part 3

Purpose

This SOP describes how to prepare a strip of DBS from a pre-collected patient sample. This SOP is to be used for sample processing of patients in the cross-sectional survey in the Malaria Control and Outreach for MMPs Along the Vietnam-Cambodia border study. These tests can be performed during the 40 minute LAMP incubation step.

Material and Equipment

- Sample collection kit
- Mechanical pipette 20-200µl (#3120000054) and disposable pipette tips (#23-0200)
- Marking pen
- 2 strips of filter paper
- Desiccant pouches
- Small zip lock bags

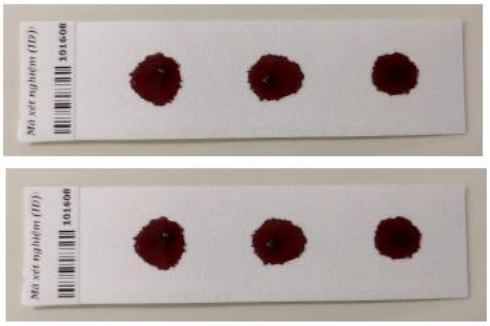
Standard operating procedure

Step-by-step actions

1. **COLLECT** a sample collection kit
2. **PREPARE** filter paper strip by attaching a barcode onto it. Place the barcode lengthwise on the left end of the filter paper
3. **TAKE** 20µl of sample from the microtainer and add it to the left side of the filter paper strip (a few cm from the label). Add a second 20µl of sample in the middle of the filter paper (a few cm from the last spot), and add a last 20µl of sample to the right side of the filter paper strip

> **Problem:** There is not enough sample to do all 6 spots of the DBS
> **Solution:** Make as many 20ul spots as you can with the sample that is left. It is ok if there is not enough for all 6 spots

4. **REPEAT** steps 2-4 on the second strip of filter paper



5. **ALLOW** strips to air dry completely (a few hours at room temperature)
6. **PLACE** a small barcode on the outside of a small zip lock bag, and place both strips of filter paper with one pouch of desiccant inside. Store at room temperature away from direct sunlight

END OF SOP FOR PROCESSING RDT, MICROSCOPY, AND DBS

SOP 6: Crossectional form

SOP6: Protocol for completing cross sectional patient form

Effective from:	TBA
Revision history:	**Authors:** – **Burnet Institute** (Leanna Surrao, Dr Vashti Irani, Duy Thanh Vo, Dr Jack Richards) **Date:** 03/09/2017 **Translated:** Nguyen Xuan Thang, Duy Thanh Vo **Date:** 03/09/17 **Reviewed by:** **Date:** **Next review due:** **Signature**(s):

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Abbreviations

SOP: standard operating procedure

LAMP: loop-mediated isothermal amplification

RDT: rapid diagnostic test

DBS: dried blood spot

Purpose

This SOP describes how complete the cross sectional patient form accurately. This SOP is to be used in the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study.

Reagents and equipment

- Pens
- Cross sectional form
- Consent form

Standard operating procedure

General guidelines

- This form is to be completed both at sample collection (questions 1-19) and sample processing sites (questions 20-41)
- The data on this form must then be uploaded to the Epicollect form "HPA4"

Step by step actions

Completed at sample collection site

1. **RECORD** the patient's name in the box provided
2. **RECORD** the patient's date of birth in the box provided. If they are unsure of the exact date, the year of birth will suffice

3. **PLACE** a tick in the appropriate box for gender
4. **PLACE** a tick in the appropriate box for ethnicity
5. **RECORD** the patient's phone number. Inform them that their phone number will only be used to contact them should we need follow up their results with them. If they are not comfortable providing their phone number, leave this section blank
6. **PLACE** a tick in the appropriate box for commune
7. **PLACE** a tick in the appropriate box for village
8. **RECORD** the patient's household code if they are aware of what it is or if it is easy to find in the code manual (located at commune health centre). If the number is not easily accessible and the patient does not know what their code is, leave this section blank
9. **RECORD** the patient's personal code if they are aware of what it is. If the patient does not know what their code is, leave this section blank
10. **EXPLAIN** the study to the patient including what the objective of the study is, how a sample will be collected from them, what the sample will be used for, and what follow-up communication they will receive. **PLACE** a tick in the appropriate box once complete
11. If the patient understands the study and agrees to participate, ask them to sign a consent form. **PLACE** a tick in the appropriate box once complete
12. **ASSESS** the clinical symptoms (if any) of the patient. Do they have symptoms of malaria such as fever? **PLACE** a tick in the appropriate box once complete
13. If "yes" was recorded for question 12, perform a rapid diagnostic test. **PLACE** a tick in the appropriate box once complete. If "no" was recorded for questions 12, skip to question 18
14. If a rapid diagnostic test was performed for this sample, check the control line. Is it present? **PLACE** a tick in the appropriate box once complete. If there is no control line, the test is invalid and must be repeated
15. Is there a test line for Pf or Pv? **PLACE** a tick in the appropriate box once complete
16. If the answer to either question 14 or 15 was "yes", refer the patient to the health care clinic. **PLACE** a tick in the appropriate box once complete
17. Collect a blood sample from the patient following the protocol detailed on *SOP2*. **PLACE** a tick in the appropriate box once complete
18. **ATTACH** a unique barcode from the sample collection kit onto the cross sectional patient form

Completed at sample processing site

19. Which commune health centre was this sample processed at? **PLACE** a tick in the appropriate box

20. When was the sample processed in a LAMP assay? This information will also be on the LAMP reaction sheet. **COMPLETE** this section using the following format "dd/mm/yy"
21. What was the run ID of this sample? This information will also be on the LAMP reaction sheet. **COMPLETE** this section using the following format "**date**(dd/mm/yy)/**time**(24 hour time)" e.g. "030917/0900"
22. Were the LAMP controls valid? **PLACE** a tick in the appropriate box
23. Was the negative extraction control valid? **PLACE** a tick in the appropriate box
24. What was the result of this sample? **PLACE** a tick in the appropriate box
25. Were there any comments about this assay? Were there any problems? **RECORD** these comments in the section provided. If there were no comments, leave this section blank
26. If there was a problem with the LAMP assay (the controls were not valid) or the sample needs to be repeated for another reason (to confirm a positive result), fill out questions 27 to 32.
27. If the sample does not need to be re-run, leave this section blank. If the sample does need to be re-run but there is insufficient sample, include a comment in section 32 stating so
28. Were 2 sets of DBS made for this sample? **PLACE** a tick in the appropriate box. If there was insufficient sample to make DBS, tick "no"
29. Was a thick smear made for this sample? **PLACE** a tick in the appropriate box. If there was insufficient sample to make DBS, tick "no"
30. What was the result of the microscopy? If the slide was not examined **PLACE** a tick in the "not done" box
31. If an RDT was already performed for this sample at the collection site, skip questions 36-40.
32. If it was not performed, perform one now.
33. Check the control line. Is it present? **PLACE** a tick in the appropriate box once complete. If there is no control line, the test is invalid and must be repeated
34. Is there a test line for Pf or Pv? **PLACE** a tick in the appropriate box once complete
35. If the answer to either question 38 or 39 was "yes", this patient needs to be followed-up. If they provided their phone number and agreed to be contacted, contact them. If they did not provide a phone number, forward their details onto the health care clinic. Record actions in the comment section of question 40
36. At the end of each day, **UPLOAD** the data into the Epicollect form "HPA4"

END OF SOP COMPLETING CROSS SECTIONAL PATIENT FORM

SOP 7: Contamination management

SOP7: Protocol for controlling contamination

Effective from:	TBA
Revision history:	**Authors:** – **Burnet Institute** (Leanna Surrao, Dr Vashti Irani, Duy Thanh Vo, Dr Jack Richards) **Date:** 12/10/2017 **Translated:** Nguyen Xuan Thang, Duy Thanh Vo **Date:** 12/10/17 **Reviewed by:** **Date:** **Next review due:** **Signature(s):**

Contact persons regarding the SOP:

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Abbreviations

LAMP: Loop-mediated isothermal amplification

MMP: Mobile migrant population

SOP: Standard operating procedure

DNA: Deoxyribonucleic acid

Purpose

This SOP describes how to prevent contamination from occurring and if it does occur, how to manage it. This SOP is to be used (if needed) while conducting the cross-sectional survey in the Malaria Control and Outreach for MMPs along the Vietnam-Cambodia border study.

Standard operating procedure

General guidelines

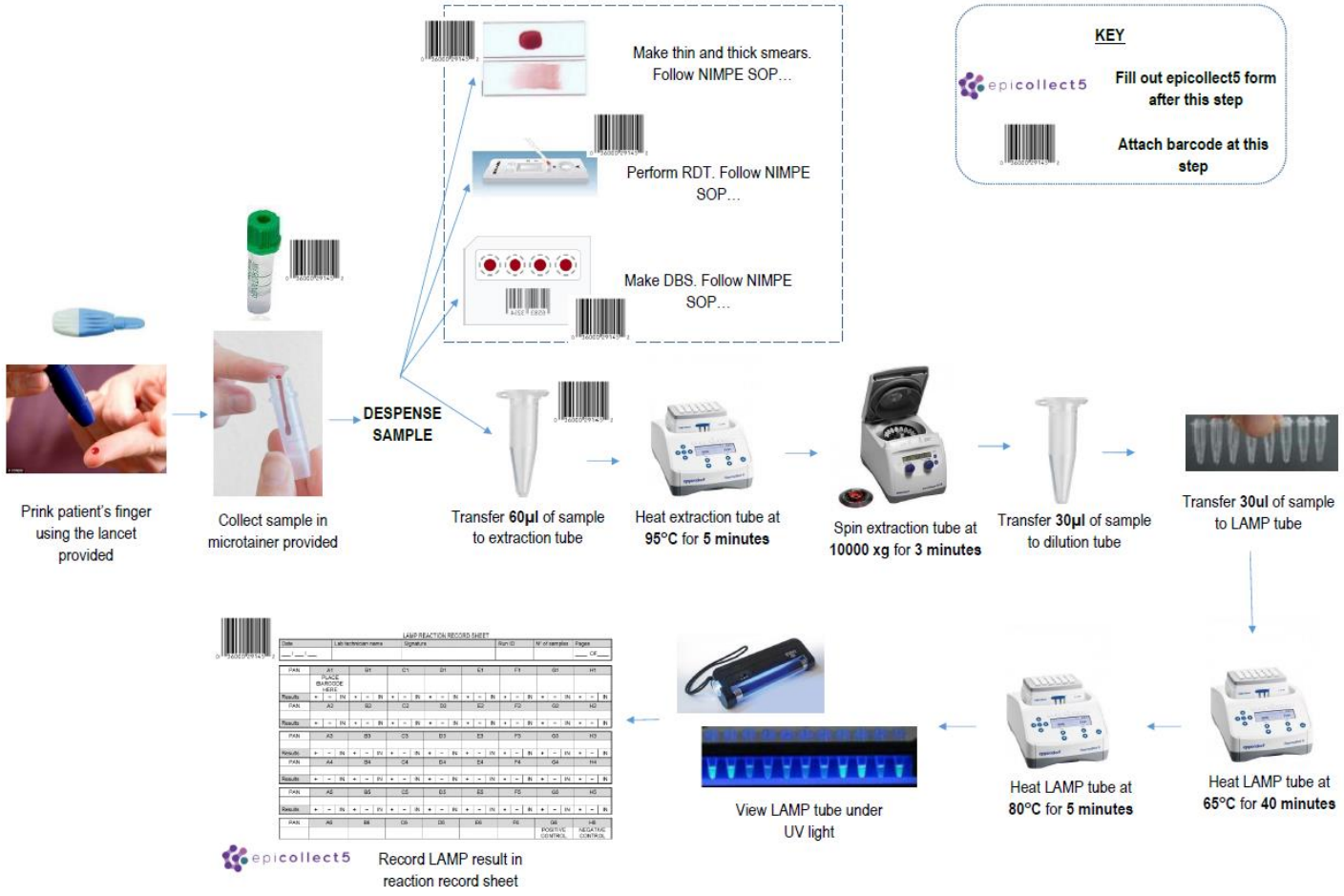
Part 1- Preventing contamination

- Never open LAMP tubes that have been amplified
- Always seal LAMP tubes in a zip lock bag just in case they open
- Dispose of consumables responsibly (e.g. used tips and tubes should be discarded immediately after use)
- Make sure blood is always contained and samples never mix
- Ensure the lids of tubes are closed firmly before heating or centrifuging
- Disinfect the bench, racks, pipettes, etc with bleach before and after use
- Always wear gloves and change them if you get blood or DNA on them
- Never leave the lids of extraction or dilution tubes open when not in use

Part 2- Controlling and eliminating contamination

1. De-contaminate everything with bleach
2. Throw out anything opened that can be replaced- i.e. pipette tips
3. It may be necessary to completely clean the pipettes. Pipettes can be dismantled and decontaminated with the right tools- contact the Burnet team if this is the case
4. Change clothes as it can carry amplified DNA and will continue to amplify
5. If contamination is severe, it may be necessary to relocate all equipment and start working somewhere else
6. After following all the above measures, run 8 negative control samples 3 times. If all samples are negative, run 4 negative controls and 4 positive controls 3 times. If all positive controls are positive and negative controls are negative, the contamination has been removed and you can continue normal LAMP sampling

Training resources: workflow summary



Appendix 3: Supplementary documents developed for RAI study

Sample collection form

This form as used to collect all relevant information from study participants. Once sample was collected, the completed form was transported and kept with the sample at all time. This information was uploaded onto an identical Epicollect form

Malaria Control and Outreach for MMPs Along the Vietnam-Cambodia Border
FORM FOR BLOOD COLLECTION AND LAMP PROCESSING FOR MALARIA
This form will be used to collect and process the samples for the cross-sectional survey.

PERSONAL INFORMATION							
1. Name:							
2. Date of Birth:							
3. Gender:	Male ☐		Female ☐				
4. Ethnicity	Kinh =1 ☐	Ê đê =2 ☐	Gia Rai=3 ☐	M'Nông=4 ☐	H'Mông=5 ☐	S'tiêng= 6 ☐	Khác=7 ☐
5. Phone number (optional):							

LOCATION					
6. Commune	Bù Gia Mập=1 ☐	Đắc Ơ=2 ☐	Đắc Nhau=3 ☐	Quảng Trực=4 ☐	Đắc Ngo=5 ☐
7. Village	Cầu Sắt =1 ☐	Bù ka =1 ☐	Đak Wi=1 ☐	Bu Prăng 1A =1 ☐	Thôn 1 =1 ☐
	Đăk Côn=2 ☐	T2-Bù Bưng =2☐	Đak Xuyên=2 ☐	Bu Prăng 2A =2 ☐	Thôn 2 =2 ☐
	Bù Lư=3 ☐	Thôn 3 =3 ☐	Thống Nhất=3 ☐	Bu Nung =3 ☐	Thôn 3 =3 ☐
	Bù Dốt=4 ☐	Thôn 4 =4 ☐	Bù Ghe=4 ☐	Bu Gia=4 ☐	Bon Điêng Đu =4 ☐
	Bù Rên=5 ☐	T2-Bù Khơn=5 ☐	Đang Lang= 5 ☐	Bu Krắk =5 ☐	Bản Đoàn Kết =5 ☐
	Bù Đăk Á=6 ☐	Thôn 6 =6 ☐	Đak Liên=6 ☐	Bu Sóp =6 ☐	Bản Tân Lập =6 ☐
	Bù La=7 ☐	Thôn 7 =7 ☐	Đak La=7 ☐	Bu Lum=7 ☐	Thôn 7 =7 ☐
	Bù Nga=8 ☐	Bù xia =8 ☐	Đak Nung =8 ☐	Bu Dăr =8 ☐	Bản Ninh Hòa =8 ☐
	Other=9 ☐	Thôn 9 =9 ☐	Other=9 ☐	Đắk Huýt =9 ☐	Bon Philte =9 ☐
		Thôn 10 =10 ☐		Bu Prăng 1 =10 ☐	Bon Philte 1 =10 ☐
		Đăk U =11 ☐		Bu Prăng 2 =11 ☐	Bản Si Át =11 ☐
		Đăk Lim =12 ☐		Other =12 ☐	Thôn Tân Bình =12☐
		Other =13 ☐			Sin Chai=13 ☐
					Giang Chau=14 ☐
					Other=15 ☐
8. Household code					
9. Personal code					

ETHICS		
10. Was the study explained to the participant?	☐ Yes	☐ No
11. Did the participant sign the consent form?	☐ Yes	☐ No

CLINICAL SYMPTOMS		
12. Does he/she have clinical symptoms of malaria?	☐ Yes – go to question **13**	☐ No, go to question **18.**
13. Did you do a Rapid Diagnostic Test?	☐ Yes	☐ No – go to question **17**
14. Is there a control line (C) ?	☐ Yes	☐ No
15. Is there a test line for *P.f* ?	☐ Yes	☐ No
16. Is there a test line for *P.v* ?	☐ Yes	☐ No
17. Did you refer the person to the health care clinic?	☐ Yes	☐ No

---------- **Sample processing in the commune health care centre** ----------

Details of the Commune HEALTH CENTRE					
20. Commune Health care centre	Bù Gia Mập=1 ☐	Đắc Ơ=2 ☐	Đắc Nhau=3 ☐	Quảng Trực=4 ☐	Đắc Ngo=5 ☐

LAMP testing				
LAMP test#1	**21.** Date of LAMP testing:			
	22. LAMP Run ID:			
	23. Was the LAMP control valid?	☐ Yes	☐ No	
	24. Is the extraction control negative?	☐ Yes	☐ No	
	25. What was the result of this sample?	☐ Positive	☐ Negative	☐ ? Unsure
	26. Comment:			
LAMP test -2 *(If problem with LAMP Test -1)*	**27.** Date of LAMP testing:			
	28. LAMP Run ID:			
	29. Was the LAMP control valid?	☐ Yes	☐ No	
	30. Was the extraction control negative?	☐ Yes	☐ No	
	31. What was the result of this sample?	☐ Positive	☐ Negative	☐ ? Unsure
	32. Comment:			
LAMP SECTION COMPLETE		☐ Yes	☐ No	

DRIED BLOOD SPOT				
33. Were Dried blood spot made for this sample?	☐ Yes	☐ No		
MICRSCOPY				
34. Was a thick smear made for this sample?	☐ Yes	☐ No		
35. Results of microscopy	☐ Positive	☐ Negative	☐ ? Unsure	☐Not Done
RAPID DIAGNOSTIC TEST				
36. Was an RDT performed for this sample?	☐ Yes	☐ No		
37. Is there a control line (C) ?	☐ Yes	☐ No		
38. Is there a test line for *P.f* ?	☐ Yes	☐ No		
39. Is there a test line for *P.v* ?	☐ Yes	☐ No		
40. Comment:				
SECTION COMPLETE	☐ Yes	☐ No		

EPICOLLECT5		
41. Is this added to EIPOCOLLECT5?	☐ Yes	☐ No

LAMP reaction record sheet

This sheet was used to record LAMP results per each batch of samples tested. An image of this form was uploaded to the Epicollect form for quality assurance purposes.

LAMP REACTION RECORD SHEET

Date	Lab technician name	Signature	Run ID	N° of samples	Pages
___ / ___ / ___					____ OF____

1			2			3			4			5			6			7			8		
BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE		
+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?
9			10			11			12			13			14			15			16		
BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE		
+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?
17			18			19			20			21			22			23			24		
BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE		
+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?
25			26			27			28			29			30			31			21		
BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE			BARCODE		
+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?
POSITIVE			NEGATIVE			BLANK			BLANK			BLANK			BLANK			BLANK			BLANK		
+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?	+	-	?

Appendix 4: Cost analysis of in-house LAMP compared to Eiken kit

During a three month study where 5000 samples are collected

Cost analysis was performed by calculating recurring and non-recurring costs based on actual expenditures encountered during the RAI study.

QUANTITATIVE ANALYSIS	YEAR 1	YEAR 2	YEAR 3	YEAR 4	YEAR 5	TOTAL
NON-RECURRING COSTS						
Pipettes	$ 1,435.50					$ 1,435.50
Microcentrifuge	$ 2,444.20					$ 2,444.20
Heat block	$ 845.00					$ 845.00
Heat block inserts	$ 400.00					$ 400.00
Pipette tips	$ 628.10					$ 628.10
Eppendorf tubes	$ 548.80					$ 548.80
Gloves	$ 1,160.00					$ 1,160.00
TOTAL NON-RECURRING COSTS	$ 7,461.60	$ -	$ -	$ -	$ -	$ 7,461.60
RECURRING COSTS						
Eiken LAMP kits	$ 72,500.00					$ -
TOTAL RECURRING COSTS	$ 72,500.00	$ -	$ -	$ -	$ -	$ -
TOTAL COSTS	$ 79,961.60	$ -	$ -	$ -	$ -	$ 7,461.60
COST SAVINGS						
Pipettes	$ 1,435.50					$ 1,435.50
Microcentrifuge	$ 2,444.20					$ 2,444.20
Pipette tips	$ 628.10					$ 628.10
Eppendorf tubes	$ 548.80					$ 548.80
Gloves	$ 1,160.00					$ 1,160.00
In-house LAMP assay	$ 4,400.00					$ 4,400.00
T8	$ 9,000.00					$ 9,000.00
TOTAL COST SAVINGS	$ 19,616.60	$ -	$ -	$ -	$ -	$ 19,616.60

QUANTITATIVE ANALYSIS	YEAR 1	YEAR 2	YEAR 3	YEAR 4	YEAR 5	TOTAL
BENEFITS						
COST SAVINGS	$ 19,616.60	$ -	$ -	$ -	$ -	$ 19,616.60
COST AVOIDANCE	$ -	$ -	$ -	$ -	$ -	$ -
REVENUE	$ -	$ -	$ -	$ -	$ -	$ -
OTHER	$ -	$ -	$ -	$ -	$ -	$ -
TOTAL BENEFITS	**$ 19,616.60**	**$ -**	**$ -**	**$ -**	**$ -**	**$ 19,616.60**
COSTS						
NON-RECURRING	$ 7,461.60	$ -	$ -	$ -	$ -	$ 7,461.60
RECURRING	$ 72,500.00	$ -	$ -	$ -	$ -	$ -
TOTAL COSTS	**$ 79,961.60**	**$ -**	**$ -**	**$ -**	**$ -**	**$ 7,461.60**
NET BENEFIT OR COST	**$ (60,345.00)**	**$ -**	**$ -**	**$ -**	**$ -**	**$ 12,155.00**

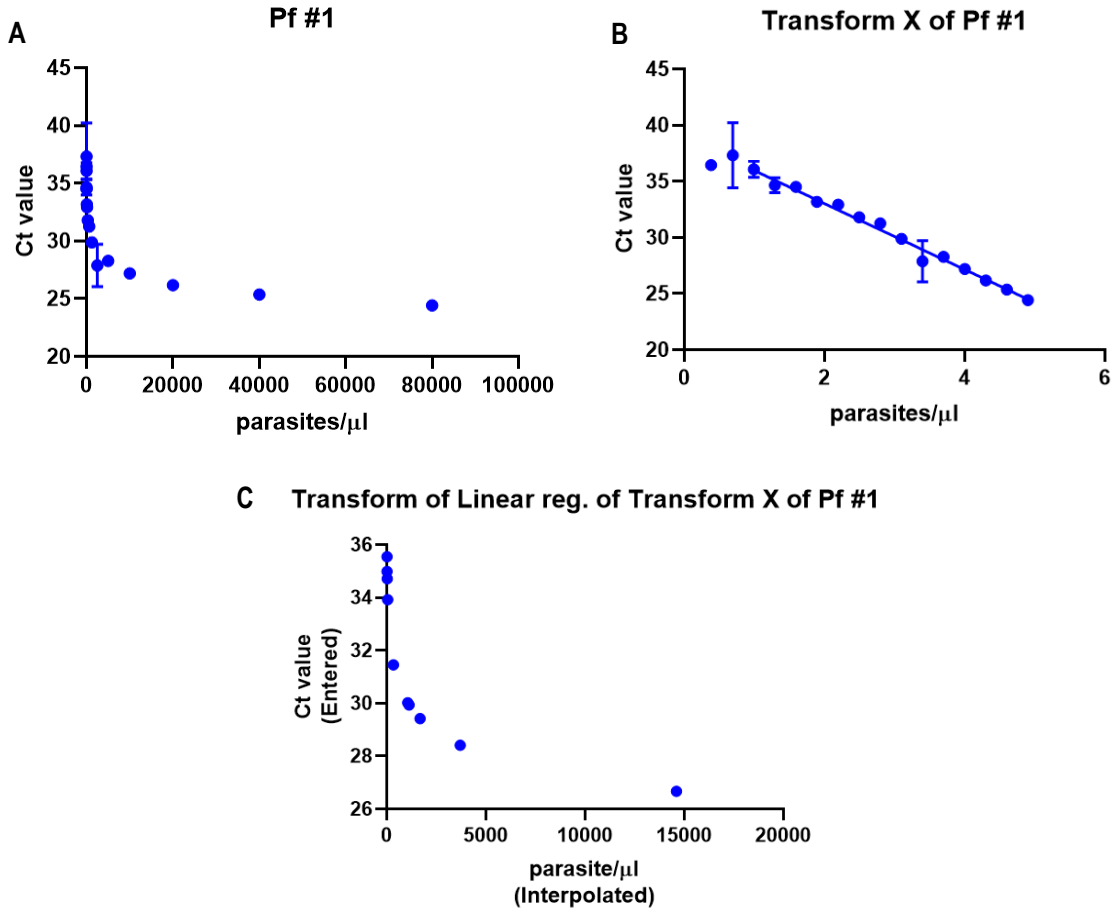
Over a 5 year period where 5000 samples are collected each year

QUANTITATIVE ANALYSIS	YEAR 1	YEAR 2	YEAR 3	YEAR 4	YEAR 5	TOTAL
NON-RECURRING COSTS						
Pipettes	$ 1,435.50					$ 1,435.50
Microcentrifuge	$ 2,444.20					$ 2,444.20
Heat block	$ 845.00					$ 845.00
Heat block inserts	$ 400.00					$ 400.00
Pipette tips	$ 628.10					$ 628.10
Eppendorf tubes	$ 548.80					$ 548.80
Gloves	$ 1,160.00					$ 1,160.00
TOTAL NON-RECURRING COSTS	$ 7,461.60	$ -	$ -	$ -	$ -	$ 7,461.60
RECURRING COSTS						
Eiken LAMP kits	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 290,000.00
TOTAL RECURRING COSTS	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 290,000.00
TOTAL COSTS	$ 79,961.60	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 297,461.60
COST SAVINGS						
Pipettes	$ 1,435.50					$ 1,435.50
Microcentrifuge	$ 2,444.20					$ 2,444.20
Pipette tips	$ 628.10					$ 628.10
Eppendorf tubes	$ 548.80					$ 548.80
Gloves	$ 1,160.00					$ 1,160.00
In-house LAMP assay	$ 4,400.00	$ 4,400.00	$ 4,400.00	$ 4,400.00	$ 4,400.00	$ 22,000.00
T8	$ 9,000.00					9,000.00
TOTAL COST SAVINGS	$ 19,616.60	$ 4,400.00	$ 4,400.00	$ 4,400.00	$ 4,400.00	$ 37,216.60

QUANTITATIVE ANALYSIS	YEAR 1	YEAR 2	YEAR 3	YEAR 4	YEAR 5	TOTAL
BENEFITS						
COST SAVINGS	$ 19,616.60	$ 4,400.00	$ 4,400.00	$ 4,400.00	$ 4,400.00	$ 37,216.60
COST AVOIDANCE	$ -	$ -	$ -	$ -	$ -	$ -
REVENUE	$ -	$ -	$ -	$ -	$ -	$ -
OTHER	$ -	$ -	$ -	$ -	$ -	$ -
TOTAL BENEFITS	**$ 19,616.60**	**$ 4,400.00**	**$ 4,400.00**	**$ 4,400.00**	**$ 4,400.00**	**$ 37,216.60**
COSTS						
NON-RECURRING	$ 7,461.60	$ -	$ -	$ -	$ -	$ 7,461.60
RECURRING	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 72,500.00	$ 290,000.00
TOTAL COSTS	**$ 79,961.60**	**$ 72,500.00**	**$ 72,500.00**	**$ 72,500.00**	**$ 72,500.00**	**$ 297,461.60**
NET BENEFIT OR COST	**$ (60,345.00)**	**$ (68,100.00)**	**$ (68,100.00)**	**$ (68,100.00)**	**$ (68,100.00)**	**$ (260,245.00)**

Appendix 5: PCR standard curve graphical transition

For quantifying PCR results, a standard curve **(A)** (fluorescence of each standard against Ct value) was transformed and analysed with a linear regression model. The transformed values of the samples were interpolated from this curve (omitting points deemed to be outside of the LOD) **(B)** and then back transformed to obtain the parasite density of samples **(C)**.



Using a standard curve to interpolate parasite densities of unknown samples. Standards used to generate this curve were known quantities of the 18s rRNA gene of *P. falciparum*.

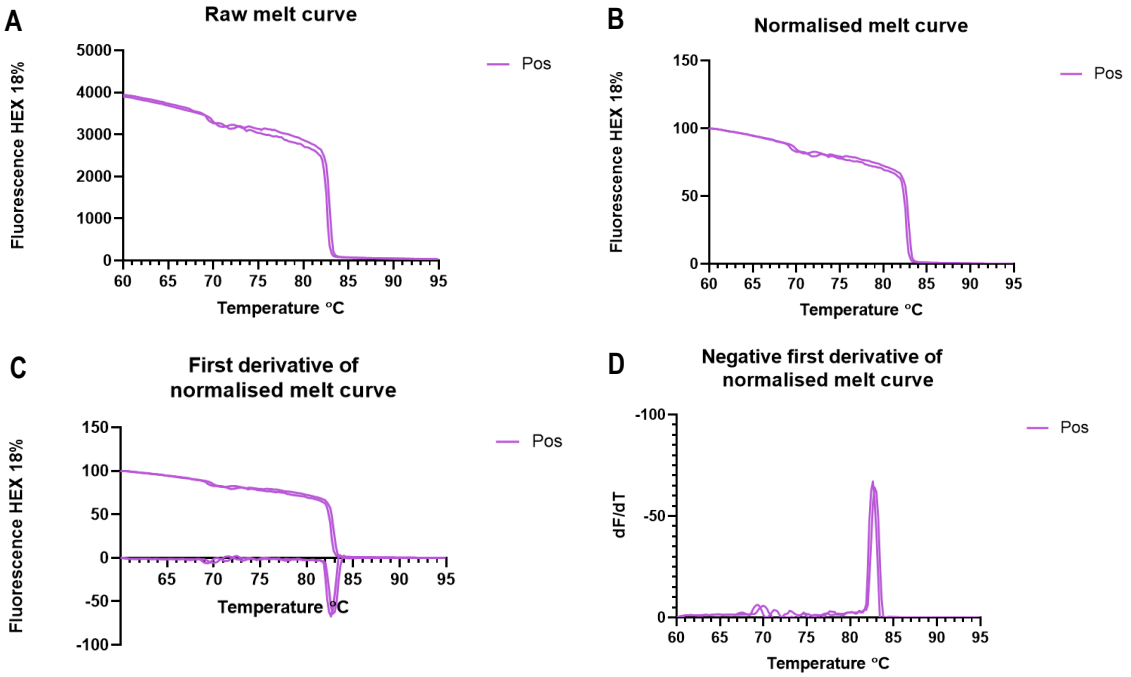
Appendix 6: Raw data used to generate LAMP positivity maps

The raw data used to generate LAMP positivity maps (Figure 4.8) can be found in the table below. This table is also present on the map itself but is illustrated here to enable clearer viewing.

Dak O village	Total positive	Total tested	% Positive
1	0	169	0.0
2	7	216	3.2
3	5	214	2.3
4	3	160	1.9
5	11	157	7.0
6	5	142	3.5
7	N/A	N/A	N/A
8	N/A	N/A	N/A
9	N/A	N/A	N/A
11	0	169	0.0
12	N/A	N/A	N/A
Bu Gia Map village	**Total positive**	**Total tested**	**% Positive**
1	0	306	0.0
2	0	374	0.0
3	5	231	2.2
4	7	220	3.2
5	15	255	5.9
6	3	224	1.3
7	0	126	0.0
8	6	217	2.8

Appendix 7: Melt curve analysis graphical transition

For melt curve analysis, curves were normalised by averaging sub-columns and normalizing means, defining 0% as the smallest value in each data set and defining 100% as the largest value in each data set. The first derivative was found from normalised curves, smoothing to four neighbours on each side of the average. The left Y axis of the figure was then reversed, showing negative values.



Generating a negative first derivative melt curve from raw melt curve data using the T8-ISO instrument. mDNA primer targets were used to amplify this *P. falciparum* positive sample

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