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Technology transfer and assay optimisation of the droplet microfluidic assay for malaria diagnostics in human saliva – A pilot study in Gabon

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Neher, Ronja Alexandra

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Dekan: Professor Dr. B. Pichler

1. Berichterstatter: Professor Dr. P. Kremsner
2. Berichterstatter: Privatdozent Dr. T. Schmidt
3. Berichterstatter: Professor Dr. A. Nüssler

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Widmung

Diese Arbeit ist meiner Mama gewidmet, die mit ihrer bedingungslosen Liebe, ihrer unermüdlichen Unterstützung, ihrem Sinn für Gerechtigkeit und ihrer grenzenlosen Toleranz und Offenheit den Grundstein für das gelegt hat, was ich bin.

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Abbreviations

#	Number
AAP	Acceptable Analytical Practice
ACT	Artemisinin-based combination therapy
AIDS	Aquired Immunodeficiency Syndrom
AIM	Action and Investment to Defeat Malaria
BSA	Bovine Serum Albumine
CERMEL	Centre des Recherches Médicales de Lambaréné
CP	Citrate phosphate
cmp	counts per minute
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytosine Triphosphate
ddPCR	digital droplet PCR
dGTP	Deoxyguanosine Triphosphate
dH_20	destilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine Triphosphate
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunoabsorband assay
EtOH	Ethanol
GTS	Global Technical Strategy for Malaria
HCl	Hydrochloride
HIV	Human Immunodeficiency Virus
HPF	High Power Fields
HRP	Histidine-rich-protein
HRP	Horseradish Peroxidase
kg	Kilogramme
LOT	batch number

Abbreviations

M	Molar
MF	Microscope Factor
ml	milliliter
mm	millimeter
NaOH	Sodiumhydroxide
nPCR	nested polymerase chain reaction
P.falciparum/vivax	*Plasmodium falciparum/viviax*
p/µl	Parasites per microliter
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
pf	*Plasmodium falciparum*
pfhrp	*Plasmodium falciparum* histidine-rich-protein
pfTopoI	*Plasmodium falciparum* topoisomerase I
pLDH	plasmodial lactate-dehydrogenase
PMSF	Henylmethylsulfonyl Flouride
RCA	rolling circle amplification
RDT	Rapid diagnostic test
REEAC	rolling circle enhanced enzyme activity detection
RNA	ribonucleic acid
ROI	Region of Interest
SN	Serial Number
SOP	Standard Operating Procedure
STARD	Standards for Reporting of Diagnostic Accuracy
TDR	Programme for Research and Training in Tropical Diseases
TE	Ethylenediaminetetraacetric acid
TMB	Tetramethybenzidine
UK	United Kingdom
USA	United States of America
USD	Dollar of the United States of America
WHO	World Health Organisation
Y/O	Years Old

I Introduction

1. Malaria

1.1 Malaria Biology, Pathogenesis and clinical features

Malaria is a mosquito-borne infectious disease prevalent particularly in tropical and subtropical regions. It is caused by protozoan parasites belonging to the genus *Plasmodium* with six species known to cause human infections. The parasite's life cycle is a complex process, which involves both human and mosquito hosts (WHO 2023b). Infection occurs through the bite of infected female *Anopheles* mosquitoes, which inject sporozoites, a specific stage of the *Plasmodium* parasite, into the bloodstream during blood feed. The sporozoites are transported to the liver, where they infect hepatocytes and within 2-10 days multiply as schizonts and release thousands of merozoites into the bloodstream. Once in the bloodstream, merozoites infect erythrocytes and undergo the intraerythrocytic lifecycle forming firstly rings, then trophozoites and eventually schizonts that develop multiple merozoites which once developed lead to rupture of the erythrocyte membrane and release of merozoites to the human blood stream (Cowman and Crabb 2006). The released merozoites then continue to infect erythrocytes. Additionally, during splenic passage, asexual parasite stages remain in the spleen initiating an endosplenic lifecycle. A proportion of merozoites form male or female sexual stages, so called gametocytes, which are essential for human to mosquito transmission. This maturation process takes place in the host’s bone marrow to bypass splenic clearance and takes about 11 days. Thereafter, gametocytes are released to peripheral circulation until up taken by a feeding mosquito. Mating occurs in the mosquito’s midgut. First zygotes are formed, then motile ookinetes invade the epithelium and develop oocysts. After asexual replication the motile sporozoites migrate to the salivary glands to be again injected into the human blood stream during the mosquito’s blood meal (González-Sanz, Berzosa et al. 2023).

While several species of *Plasmodium* are capable of infecting animals, the most common and clinically significant ones infecting humans include *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale wallikeri, Plasmodium ovale curtisi and Plasmodium malariae (Centers for Disease Control and Prevention CDC 2020).* Additionally, *Plasmodium knowlesi*, which mainly infects primates, gained more attention during recent years due to zoonotic malaria infections and is ultimately recognized as human pathogenic malaria species (Anstey, Grigg et al. 2021). The complex interplay between the human host and the different *Plasmodium* species defines the pathogenesis of this disease, leading to the characteristic clinical features that range from mild flu-like symptoms to severe complications, including organ failure and death (Poespoprodjo, Douglas et al.). Understanding the intricate molecular and cellular mechanisms underlying the interactions between the parasite and the host is crucial for developing effective prevention and treatment strategies.

Plasmodium falciparum is responsible for the most severe form of malaria and is the deadliest of all Plasmodium species. The WHO reports that *P. falciparum* is responsible for most malaria-related deaths globally (WHO 2023d). This species is prevalent in tropical and subtropical regions, with a high burden of disease in sub-Saharan Africa. *P. falciparum* infections can lead to severe clinical manifestations, including shock, renal injury, cerebral malaria, severe anaemia, and multi-organ failure, thrombocytopenia, respiratory distress, often resulting in death (Poespoprodjo, Douglas et al.). Its ability to sequester in the microvasculature of various organs and adherence of red blood cells to endothelial cells leads to microvascular obstruction and local inflammatory responses. Its rapid multiplication within red blood cells contributes to the acute clinical manifestation (Cowman, Healer et al. 2016). The dysregulation of immune responses further exacerbates the severity of the disease, with the host's immune system both attempting to control the infection and contributing to tissue damage.

Plasmodium vivax is another common human malaria parasite, with a broader geographic distribution compared to *P. falciparum. P. vivax* is prevalent in Asia,

South America, and some parts of Africa. While it generally causes a less severe form of malaria, it can result in relapses due to its ability to form dormant liver stages (hypnozoites) (Price, Tjitra et al. 2007). These reactivations and blood stream invasions can occur even years after the infecting mosquito bite. The pathogenesis of *P. vivax* involves the rupture of infected red blood cells and the sequestration of merozoites in various tissues, often leading to severe anaemia and splenic rupture, albeit less frequently than *P. falciparum.* Additionally, the propensity of *P. vivax* to invade reticulocytes influences disease severity and complicates treatment strategies.

Plasmodium ovale walliki and *Plasmodium ovale curtisi* are distributed throughout the malaria endemic world (Collins and Jeffery 2005). Infections with *P. ovale walliki and curtisi* can result in a mild form of malaria, they have a unique relapse pattern and may persist in the liver for extended periods.

Plasmodium malariae, on the other hand, is widespread in many parts of the world, but infections are typically less frequent than those caused by *P. falciparum* or *P. vivax* (Centers for Disease Control and Prevention CDC 2020). *P. malariae* infections can persist for many years, and chronic malaria with low-grade parasitaemia is a characteristic feature. Though it is usually a milder form of malaria, complications such as nephrotic syndrome and glomerulonephritis have been associated with *P. malariae.*

Plasmodium knowlesi can be found in Southeast Asia and typically infects macaques (Anstey, Grigg et al. 2021). It has been subject to zoonotic malaria infections especially in Malaysia. Due to its short replication cycle of 24 hours, it has the ability to fast progression and potentially fatal outcome. Pathogenesis of *Plasmodium knowlesi* is similar to *P. falciparum*, involving the invasion of red blood cells by the parasite, leading to haemolysis and triggering inflammatory responses. The cytoadherence of infected red blood cells to endothelial cells contributes to microvascular sequestration. Key clinical symptoms include fever, chills, and headache, resembling other malaria species. Notably, severe cases

with organ dysfunction, respiratory distress, and neurological complications were observed.

The parasite's vector is the *Anopheles* mosquito, which is present in all continents except the Antarctic region with particular species being dominant in different geographic regions (Centers for Disease Control and Prevention CDC 2020). Out of the about 430 known *Anopheles* species, 30-40 are able to transmit malaria due to the fact that for the development of different stages of malaria parasites in the *Anopheles* mosquito, specific environmental factors such as high temperatures and humidity are essential. While male *Anopheles* mosquitos feed on sugar sources, female mosquitos depend upon blood meals for egg development and are therefore responsible for the transmission of malaria. Besides the possible threat of malaria transmission and infection in malaria-endemic regions, imported malaria may introduce the parasites into non-endemic regions (Weiland 2023). Imported malaria cases occur when individuals acquire the infection in endemic regions and subsequently travel to non-endemic regions, where transmission does not naturally occur. Notably, globalization and human mobility in terms of travel and migration has been identified as a key driver of imported malaria (González-Sanz, Berzosa et al. 2023). These cases have been documented in non-endemic regions such as the Unaided Staes of America, Greece, France, and Belgium, and they represent a substantial public health concern due to the potential for local onwards transmission via local Anopheles mosquito vectors (González-Sanz, Berzosa et al. 2023). Furthermore, airport malaria is a rare but often overlooked mode of transmission of the disease. This occurs when infected mosquitoes stow away on aircraft or luggage and can lead to transmission events in non-endemic regions (Alenou and Etang 2021).

Over all, clinical features of malaria are diverse and can range from mild flu-like symptoms to severe, life-threatening complications (Cowman, Healer et al. 2016). Fever is the hallmark symptom, often accompanied by chills, sweats, headache, and myalgia. Thrombocytopenia, anaemia, and hepatosplenomegaly are common haematological manifestations. Cerebral malaria mainly associated

with *P. falciparum* malaria, is characterized by altered consciousness, seizures, and neurological deficits. The distinct clinical presentation of each Plasmodium species reflects variations in parasite biology, host immune responses, and the overall pathogenicity of the infection.

1.2 Malaria treatment

The cornerstone of malaria treatment remains artemisinin-based combination therapies (ACTs), which combine an artemisinin derivative with a partner drug to enhance efficacy and delay the emergence of resistance (WHO 2023c). Artemisinins, derived from the sweet wormwood plant (Artemisia annua), rapidly reduce parasitaemia, providing a swift and potent antimalarial effect (Krishna, Bustamante et al. 2008). The World Health Organization recommends the use of ACTs due to their efficacy in reducing parasite burden and the development of resistance (WHO 2023c). Dihydroartemisinin-piperaquine and artemether-lumefantrine are two widely utilized ACTs, with documented success in various endemic regions. For severe malaria, parenteral application of artensunate, is the recommended first-line treatment (White 2022). In addition to artemisinin derivatives and artemisinin combination therapies, other classes of antimalarials play role in the treatment of malaria. Chloroquine, once a mainstay, has faced challenges due to widespread resistance, particularly in *Plasmodium falciparum*. Therefore it is not used anymore on a wide scale for the treatment of Malaria tropica (Poespoprodjo, Douglas et al.). Quinine remains an alternative treatment option for severe malaria and for drug resistant falciparum malaria, foremost when used in combination with antibiotics with antimalarial activity such as tetracyclines and clindamycin (WHO 2023c). The 8-aminoquinoline drugs primaquine and tafenoquine are used for radical cure treatment of Malaria tertiana by targeting hypnocoite stages. While primaquine requires repeated prolonged intake, tafenoquine's long half-life allows for a single-dose regimen, improving adherence (Llanos-Cuentas, Lacerda et al. 2014). Supportive therapy may involve fluid therapy, blood transfusion in case of severe anaemia,

anticonvulsive therapy for patients presenting with seizures due to cerebral malaria, intubation and mechanical ventilation for comatose patients. Children with severe malaria often present with bacterial co-infections and benefit from addition antibiotic therapy (Poespoprodjo, Douglas et al.),(WHO 2022).

1.3 Global burden of malaria

Malaria is a potentially life-threatening parasitic disease transmitted by the bites of infected female Anopheles mosquitoes (WHO 2023d). The majority of malaria cases is reported in tropical and subtropical climatic zones of Africa and to a lesser degree in Asia and South America. In 2022 malaria was endemic in 85 different countries. Like in previous years, in 2022 the highest disease burden was carried by the World Health Organisation (WHO) African Regions, where approximately 233 million (94%) of the 249 million global malaria cases occurred. Between 2000 and 2019 the number of malaria deaths in the WHO African Regions was reduced by 44% and an overall steady global decline in malaria deaths was reported for the period between 2000 and 2019. However, in 2020 the number of malaria deaths increased again by 10% in comparison to the previous year due to interrupted health care services caused by the COVID-19 pandemic. In 2021 and 2022 a slight decrease in malaria deaths could be seen, yet 580,000 deaths caused by malaria were recorded in the WHO African Regions in 2022, leading to a total of 608,000 malaria deaths worldwide (WHO 2023d).

During the past decades, the development and implementation of malaria control interventions as well as multinational guidelines and action plans such as the *Global Technical Strategy for Malaria 2016–2030 (GTS) (GTS 2021)* and the *Action and Investment to defeat Malaria 2016-2030 (AIM)* published by the Roll Back Malaria Initiative (Nabarro 1999) with their global targets and milestones were huge steps towards the vision of malaria elimination.

Malaria disproportionately affects low-income countries. This disparity arises from a complex interplay of socioeconomic, environmental and healthcare factors leaving the most vulnerable nations at highest risk (Ebhuoma, Gebreslasie et al.

2022). Low-income countries often lack the financial resources necessary to invest in comprehensive malaria control programs, including the distribution of insecticide-treated bed nets, access to healthcare facilities and essential antimalarial drugs (WHO 2019). Furthermore, limited healthcare infrastructure in affected countries including shortages of healthcare workers and essential medicines hinder diagnosis and treatment of malaria, resulting in delayed or inadequate care for those affected (Feachem, Chen et al. 2019). In addition, political and social instabilities as for example seen recently in Venezuela (Grillet, Hernández-Villena et al. 2019) or Myanmar (Rae, Nosten et al. 2022), can disrupt healthcare delivery systems and divert resources away from public health efforts, hampering malaria control efforts and exacerbating the disease's impact. Environmental factors such as inadequate sanitation and housing, which create conducive breeding grounds for malaria-transmitting mosquitoes aggravate transmission in low-income countries additionally (WHO 2022).

The WHO estimates that in 2022 still about half of the world’s population was residing in regions at risk for malaria infections (WHO 2023d), which once more emphasises the importance of constant development towards malaria control.

1.4 Distribution and impact of financial investments

To fight malaria, 3.5 billion USD were spent globally on infection control and elimination in 2021, a slight increase compared to previous years (3.3 billion USD in 2020, 3.0 billion USD in 2019). Between 2010 and 2021 about two thirds of the total funding for disease control came from international sources, mainly the USA, UK, France and Germany. Governments of endemic countries covered about one third of total expenses for malaria control by themselves in 2021 (Feachem, Phillips et al. 2010), (WHO 2022). While the total investment spent on malaria control increased continuously during the last decade, the GTS claims that annual investment should rise to 9.3 billion USD by 2025 and even 10.3 billion USD by 2030 in order to reach adequate widespread coverage of possible interventions. Despite the already existing funding gap, the COVID-19 pandemic has led to an

additional financial deficit in many countries, whose impact is not yet fully understood (Patouillard, Griffin et al. 2017), (WHO 2015b).

In 2021, 626 million USD were available for malaria research, a declining sum for the third successive year. The reallocation of funds and research priorities towards COVID-19 may have inadvertently diverted resources away from ongoing malaria research efforts. However, since 2007, the funding for malaria research is along with funding for HIV/AIDS and tuberculosis one of the top three fund receiver diseases (G-Finder 2023). Nevertheless, annual funding of about 851 million USD would be required according to the GTS during the 2021 to 2030 period to meet 2030's milestones concerning malaria elimination. Main funders for research investments are the US National Institutes of Health as largest investor, accompanied by the Bill & Melinda Gates Foundation, the European Union and industry (WHO 2022), (G-Finder 2021), (WHO 2020).

The impact of invested funds is enormous for local progress in malaria control. It is described that countries receiving significant funding for research are often those nations where high investments in malaria control are made (Adegnika, Honkpehedji et al. 2021), (Goenka and Liu 2020), (Magalhães, Codeço et al. 2023). Vice versa, countries with relatively low investment in control measures are also less likely to receive generous funding for research. The latter countries are frequently areas with high disease burden, high malaria related mortality and weak infrastructures as well as inner political instabilities. A relationship between a country's socio-economic situation, the ability to invest into malaria research, the implemented disease control standards and the amount of received funds can be observed (Head, Goss et al. 2017). Calculations disclose that the 20 countries with highest malaria burden will need 88% of the total investments for disease control to be able to comply with the *Global Technical Strategy for Malaria (GTS)* goals (Patouillard, Griffin et al. 2017). For given reasons, the *Action and Investment to Defeat Malaria (AIM*) considers malaria as not only a health problem but evaluates malaria in a *"developmental, economic, political, security,*

environmental, agricultural, educational, biological and social" (WHO 2015a) context. Moreover, through reduction of malaria related morbidity, a progress in almost all sustainable development goals set by the United Nations is expected.

For successful malaria elimination, several points of action have to be taken in account. The *Global Technical Strategy for Malaria (GTS)* has set its focus on three pillars: Firstly, ensuring global access to malaria prevention, diagnostics and treatment; secondly, supporting the worldwide process of malaria elimination and thirdly, the development of effective malaria surveillance (GTS 2021). The WHO suggests a so-called package of tools for high transmission countries, which lists different key interventions to achieve reduced malaria case incidence and mortality. This package of tools highlights the importance of disease prevention, vector control, confirmation of diagnosis, correct and immediate treatment and malaria surveillance systems (WHO 2015a), (Sabot, Cohen et al. 2010), (Wirth, Casamitjana et al. 2018). More detailed, the three basic components of the described toolkit recommended for national malaria control programs are long-lasting insecticide-treated bed nets to prevent transmission, fast-acting antimalarials to treat and rapid diagnostic tests to detect and confirm infections (Zimmerman and Howes 2015).

As depicted on Figure 1, funding for research and development is distributed very unequally and changed only little over the past years. Research areas such as drug development, basic research as well as vaccine advancement received intense funding on high levels while funding for malaria diagnostic methods obtained only 2.5% of the total malaria funding in 2021 (WHO 2022).

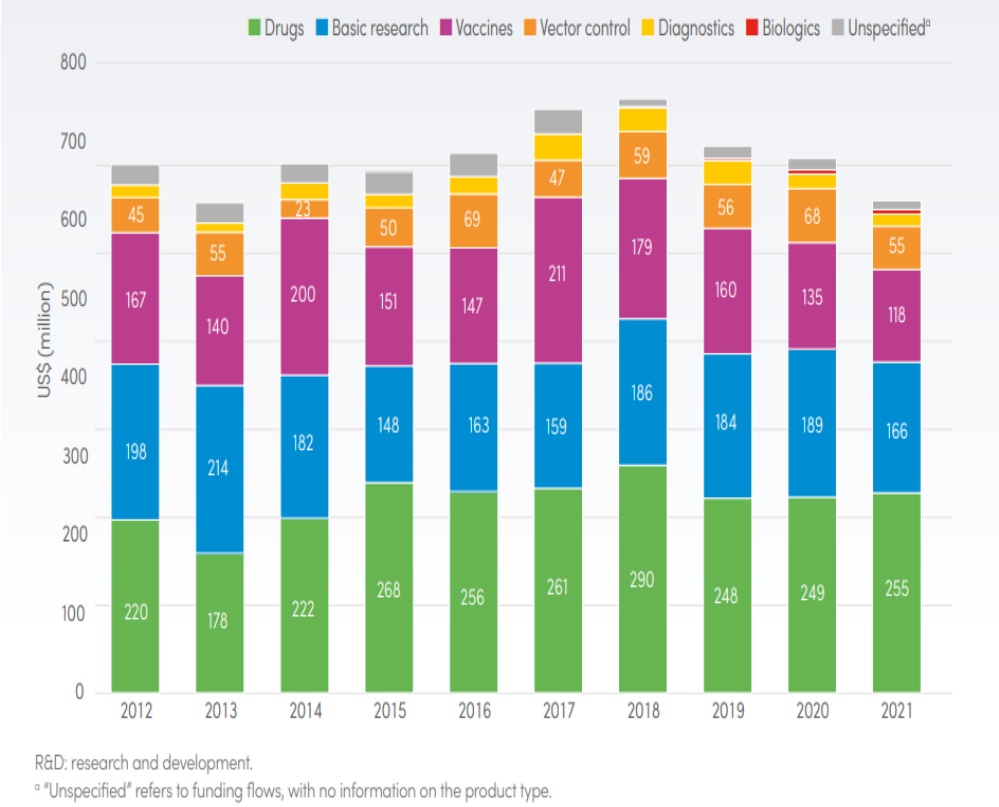


Figure 1 Funding for malaria-related R&D, 2012–2021, by product type (constant 2021 US$)

Source: World malaria report 2022. Geneva: World Health Organization; 2022. Licence:CC BY-NC-SA 3.0 IGO.

2. Established malaria diagnostic methods

2.1 Blood smear microscopy

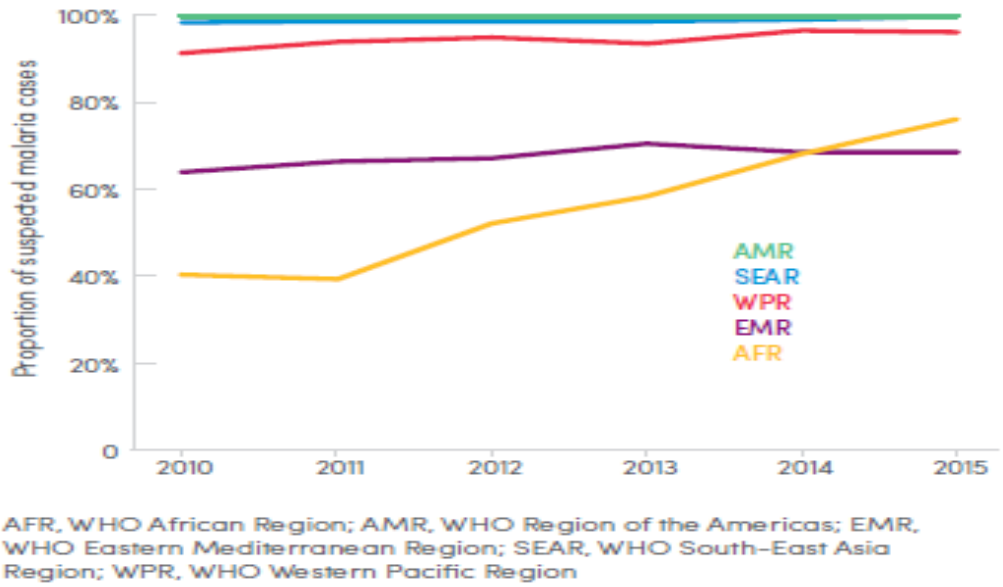
Since the microscopic examination of blood allows the detection of all Plasmodium species, is applicable for low parasitaemia samples with its detection limit of around 10 parasites/µl in expert microscopy and approximately 100 parasites/µl in routine microscopy (Joanny, Löhr et al. 2014), (Payne 1988) and makes a quantification of the parasites possible, the thick and thin blood smear microscopy has numerous advantages over other diagnostic assays and remains the clinical gold standard for confirming malaria diagnosis (Chotivanich, Silamut

et al. 2007). However, it constitutes a reader dependent and subjective method, which requires appropriate laboratory staff training. In addition, microscopy causes a delay in result reporting as slides have to be prepared, dried and coloured before diagnostics can be performed. Moreover, an initial investment into laboratory equipment such as microscopes and staining has to be made (Mathison and Pritt 2017).

2.2 Rapid diagnostic tests

Another diagnostic method of malaria diagnostics are rapid diagnostic tests (RDTs). Malaria RDTs are based on antigen detection by monoclonal antibodies which are framed in lateral flow assays in a simple card format. Detected antigens include histidein-rich-protein-2, parasitic lactate dehydrogenase or aldolase (Moody 2002). During the past years, RDTs gained importance and changed the availability of malaria diagnostics significantly (WHO 2017). Between 2010 and 2022, 3.9 billion RDTs were sold globally (WHO 2023d). RDTs are easy to handle and require only very little training of performing personnel and due to their handy format and limited number of reagents they depend upon, RDTs can be easily used outside health care facilities (Mathison and Pritt 2017). Other than that, the test result can be seen immediately, which makes RDTs an important point-of-care instrument. Rapid tests are also relatively affordable with an average piece price between 0.2 and 1.0 USD and are, compared to microscopy, the most cost-effective malaria diagnostic method (Batwala, Magnussen et al. 2011). Between 2010 and 2015, the percentage of patients with suspected malaria receiving a diagnostic test has increased from 10% to 76% in the WHO African regions as shown on Figure 2. Among these performed tests, RDTs presented 74% of all diagnostic methods, which shows their widespread implementation (WHO 2016a). In a contemporary meta-analytical examination encompassing research spanning the years 2009 to 2023, predominantly undertaken within the African context, congruent outcomes were observed. Azizi et al. (2023) delineate a comprehensive prevalence rate of Rapid Diagnostic Tests (RDTs) amounting to 76%(Azizi, Davtalab Esmaeili et al. 2023). Besides the advantages which rapid

diagnostic tests offer, there is also a down side to their use. Firstly, RDTs do not provide a quantitative test result. In addition, their performance regarding the detection limit is somewhat poorer than microscopy. It was shown that conventional RDTs evaluated by the WHO have an average detection limit of 200 – 2000 parasites per microliter (WHO 2014a). Finally, mixed species infections and infections by non-falciparum isolates are less well detected by RDTs than *Plasmodium falciparum* infections.



AFR, WHO African Region; AMR, WHO Region of the Americas; EMR, WHO Eastern Mediterranean Region; SEAR, WHO South-East Asia Region; WPR, WHO Western Pacific Region

Fig. 2 Proportion of patients with suspected malaria infection receiving a RDT at a public health care facility
Especially in the African Regions, the percentage of patients receiving a RDT to confirm suspected malaria diagnoses increased between 2010 and 2015

Source: World Malaria Report 2016. Geneva: World Health Organization; 2016. Licence: CC BY-NC-SA 3.0 IGO

Lately, ultrasensitive RDTs (uRDT) with, at least under laboratory conditions, 10-fold lower detection limit than conventional RDTs (cRDT) were developed to improve the identification of low-density, asymptomatic malaria infections (Owalla, Okurut et al. 2020).

2.3 Molecular methods

With subclinical infections being a major obstacle to effectively control malaria in low transmission areas, more molecular methods are constantly being developed to detect even lower parasite levels. It was estimated by Slater et al. (2015) that by increasing the diagnostic sensitivity from 200 parasites/µl to 2 parasites/µl, 95% percent of the infectious reservoir could be detected instead of 55% only (Slater, Ross et al. 2015). Based on nucleic acid amplification tests (NAAT), different methods of PCRs are available, such as the quantitative real-time PCR (qPCR) and the quantitative reverse transcriptase PCR (RT-qPCR) both with parasite detection limits <1 parasite/µl (Mangold, Manson et al. 2005). In many studies, qPCR is used as reference diagnostic method when evaluating new diagnostic tests. Nevertheless, the performance of PCR requires trained laboratory staff members and adequate equipment and furthermore is time consuming.

The loop-mediated isothermal amplification (LAMP) is another molecular diagnostic method, which is easy to handle, fast and may lead to a parasite detection limit of around 2 parasites/µl (Rypien, Chow et al. 2017). It is a WHO recommended diagnostic tool for epidemiological surveys, mass screening and treatment and focus investigations in low-transmission settings for microscopic malaria (WHO 2014b).

Another molecular assay is a method based on recombinase polymerase amplification (RPA), which can be performed at a constant and low temperature with simple and portable equipment. Combined with lateral flow strips for readout, the RPA assay for DNA detection is potentially feasible for low-resource settings (WHO 2014a). Lalmemruata et al. (2020) recently further developed this method using a reverse transcriptase RPA resulting in similar results as RT-qPCR with more than 90% accuracy in the detection of *Plasmodium falciparum* parasites and therefore consistently above the performance level of LAMP and furthermore involving a minimal amount of chemicals and readout on lateral flow strips visible after 10 minutes (Lalremruata, Nguyen et al. 2020).

2.4 Emerging diagnostic methods

Digital microscopy, coupled with image analysis algorithms, represents another promising avenue for improved malaria diagnosis. Automated image analysis software can rapidly scan and analyse blood smears and identify malaria parasites, assisting healthcare professionals in parasite detection (Maturana, de Oliveira et al. 2022). This approach improves diagnostic efficiency and minimizes human error. Smartphone-based microscopes have been developed that can capture high-resolution images of blood smears for malaria diagnosis. This approach aims to improve accessibility to diagnostic tools, especially in resource-limited settings. However, challenges persist in standardizing image acquisition and analysis protocols (Dey, Nath et al. 2021). Emerging technologies in biosensors have led to the development of novel diagnostic tools for malaria. Biosensors, incorporating specific receptors for Plasmodium biomarkers, offer rapid and sensitive detection (Krampa, Aniweh et al. 2020). A biosensor is a device that integrates a biological sensing element with a transducer to detect and convert a biological response into an electrical signal. In the context of malaria diagnostics, biosensors can be designed to detect specific biomarkers associated with the presence of the malaria parasite such as antigens or genetic material from the *Plasmodium* parasites. Common biomarkers include histidine-rich protein 2 (HRP2), lactate dehydrogenase (LDH), and Plasmodium DNA. The binding of the biomarker to the biorecognition element generates a signal that is transduced into a measurable output, typically an electrical signal. The electrical signal is then processed and analysed to determine the presence and concentration of the malaria biomarkers (Singh, Thungon et al. 2019, Huang, Zhang et al. 2023).

Further indirect detection methods are more likely to be used for epidemiological studies and in research settings but are less applicable for acute malaria diagnostics. Illustratively, the antibody-based enzyme-linked immunosorbent assays (ELISA) which can monitor the host's immune response to malaria infections or flow cytometry for species identification (Fitri, Widaningrum et al. 2022).

2.5 Considerations on the appropriate biological samples for malaria diagnostics

Conventional malaria diagnostic methods including microscopy and RDTs require blood specimens. Blood samples are commonly obtained by venepuncture or a finger prick, since only small volumes of blood are needed for diagnostic assays (Zimmerman and Howes 2015). However, blood sampling involves various risks for both the subject undergoing a blood draw or finger prick and the person performing the sampling. In order to minimize the risks arising with blood sample collection and patient discomfort, alternatives to blood testing are being evaluated. Recently, saliva and urine have come into focus as diagnostic media for malaria diagnostics. Tao et al. (2019) could identify 35 malaria parasite markers in human saliva, one of them being a protein specific to female gametocytes found in saliva of children with subclinical malaria infection (Tao, McGill et al. 2019). Several studies reveal the presence of malaria parasite related components and the *Plasmodium* parasites genetic products in human saliva. For example, HRP-2 has been detected by ELISA with similar sensitivities and specificity in human blood and saliva (Wilson, Adjei et al. 2008), (Fung, Damoiseaux et al. 2012). Additionally, the detection of Plasmodium falciparum lactate dehydrogenase (pfLDH) has been feasible through rapid diagnostic tests (RDTs) in both native and centrifuged saliva samples (Gbotosho, Happi et al. 2010) and the presence of a ribosomal RNA subunit of the parasites has been ascertained through nested polymerase chain reaction (PCR) in patients infected with *Pasmodium falciparum* and/or *viv*ax (Ghayour Najafabadi, Oormazdi et al. 2014), (Singh, Singh et al. 2014). Moreover, Esteves et al. (2011) have demonstrated a discernible correlation between malaria antibody levels in plasma and saliva (Estévez, Satoguina et al. 2011). The efficacy of diagnosing malaria from urine has been substantiated through successful demonstrations in several studies employing methodologies analogous to those used for saliva testing. Utilizing nucleic acid-based techniques such as nested polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), the identification of plasmodial ribosomal RNA and mitochondrial genes within urine

has been achieved. However, it is noteworthy that the sensitivity of these methods in urine analysis has been observed to be inferior when compared to their application in saliva analysis (Putaporntip, Buppan et al. 2011), (Ghayour Najafabadi, Oormazdi et al. 2014). In contrast to diagnostic modalities focusing on saliva, a commercially available kit specifically targeting the histidine-rich protein-2 (HRP-2) antigen in urine has been developed for malaria diagnosis. Oyibo et al. (2017) conducted a study in Nigeria, revealing a sensitivity of 79% and specificity of 89% in comparison to conventional blood-based RDTs (Oyibo, Ezeigwe et al. 2017). Similar findings were reported by Godwin et al. in 2018 (Godwin A.A. 2018). However, Oyeinyi et al. (2022) presented contrasting outcomes, demonstrating markedly poorer performance of the urine-based malaria rapid diagnostic test (Oyeniyi, Bello et al. 2022). Limitations of the urine malaria diagnostic test are its relatively high detection limit of 125 parasites/µl blood of corresponding blood samples as well as their exclusive capability to detect *Plasmodium falciparum* (Oyibo, Ezeigwe et al. 2017).

3. Development and evaluation of diagnostic tests

Bearing the *Global Technical Strategy for Malaria* with its aims in mind and concerning the outcomes of recent studies described above, highly sensitive and specific point-of-care diagnostic tests, which can diagnose malaria precisely and rapidly, are urgently needed to further reduce malaria related morbidity and mortality (GTS 2021).

Since the evaluation of diagnostics is absolutely essential on one hand, but on the other hand it was found that neither the quality of the conducted studies nor the quantity was sufficient, an initiative was being developed to optimise the standard of evaluating and reporting on diagnostic testing. The *Standards for Reporting of Diagnostic Accuracy* (STARD) statement is a checklist of 25 items, which is supposed to guide researchers and ensure that all relevant data

concerning a diagnostic test under evaluation is being recorded (Bossuyt, Reitsma et al. 2003). Categories that should be evaluated according to STARD are, among others, the test performance, the ease of use, the conditions of use and storage as well as the shelf life In addition, the WHO has set up more specific requirements for the validation of malaria RDTs, being a minimum detection limit of 100 parasites per microliter, a minimum sensitivity of 95% and a specificity of at least 90% (Mouatcho and Goldring 2013).

In 2006, the Programme for Research and Training in Tropical Diseases (TDR) published a series on evaluating diagnostics. The authors state that information about whether which test is appropriate and useful in a specific setting has to be tested via an evaluation of the given assay either in a laboratory or a field setting. The process of test development described as bench-to-bedside-pathway involves several consecutive steps, as shown on figure 3 (Peeling, Smith et al. 2006).

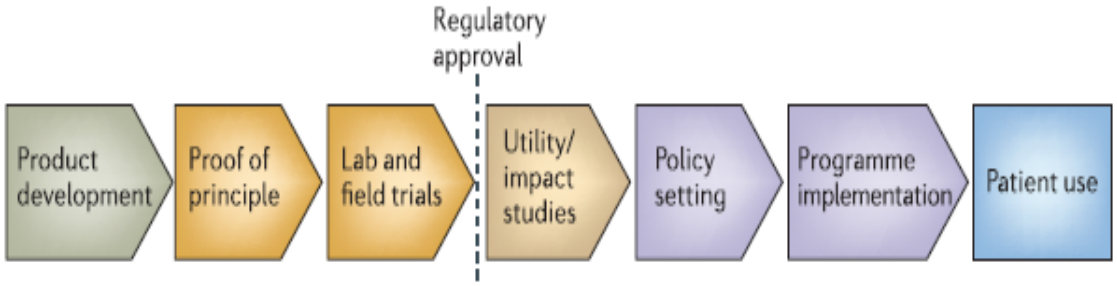


Fig. 3 The bench-to-bedside pathway of diagnostics development and evaluation.
The development of a diagnostic test usually follows a path from identification of the diagnostic target and optimization of test reagents to the development of a test prototype that then undergoes a series of evaluations

Source: Peeling, R. W., Smith, P. G., & Bossuyt, P. M. (2006). A guide for diagnostic evaluations. Nature reviews. Microbiology, 4(12 Suppl), S2–S6. https://doi.org/10.1038/nrmicro1568

The WHO states that technology transfer is “the transfer of technical information, tacit know-how, performance skills, technical material or equipment, jointly or as individual elements, with the intent of enabling the technological or manufacturing capacity of the recipients” (WHO 2012). It is further explained that technology transfer is the collaboration of knowledge and resources that aims to develop new

medical devices in favour of public health needs. Its purpose is to qualify the receiving laboratory to carry out a certain analytical method.

The guidance document Acceptable Analytical Practice AAP introduces three different approaches to technology transfer, being either comparative testing of the transferring and receiving laboratory or partial or full validation of analytical parameters (Okamoto 2014). The latter approach is applicable for the technology transfer of the microfluidic assay – a full validation of its sensitivity, specificity, quality and utility was planned to be conducted in Gabon in a field study.

3.1 The REEAD method

As described above, a new diagnostic approach constitutes analysing saliva as diagnostic medium in order to increase patient comfort and to facilitate (repeated) sample collection. Recently, a new method of processing human saliva has been proven to successfully detect the *Plasmodium* parasites active enzyme topoisomerase I (Juul, Nielsen et al. 2012). This new assay combines a hand driven enzyme extraction from saliva with a specific amplification method called rolling circle amplification (RCA) to visualize enzyme activity (RCA enhanced enzyme activity detection or REEAD). A study conducted in Denmark showed successful malaria detection in 33 out of 33 saliva samples from infected individuals. While the sampling for this project was done in a malaria endemic area, the test itself was performed in Denmark (Hede, Fjelstrup et al. 2018). The key element of the REEAD is the presence of the *Plasmodium* topoisomerase I (pfTopoI), an enzyme expressed by the malaria parasite. Generally, DNA topoisomerases mediate relaxation of topological tension generated during important cellular processes such as replication and transcription in DNA by catalysing the cutting and ligation of DNA strands. Therefore, these enzymes play an important role in DNA replication, recombination and repair mechanisms within an organism's life cycle (Cortopassi, Penna-Coutinho et al. 2014). While topoisomerase class I enzymes cut single DNA strands, class II topoisomerases introduce breaks in both strands of double stranded DNA (Tosh and Kilbey 1995).

After the insertion of breaks into DNA strands, the re-joining is achieved by a covalent DNA-enzyme intermediate (Juul, Nielsen et al. 2012). The described topoisomerase characteristics are used in the REEAD, where the plasmodial topoisomerase I converts self-folding oligonucleotide DNA substrates into covalently closed DNA circles, which are in turn substrates for the rolling circle amplification in order to visualize the enzymatic activity (Tesauro, Juul et al. 2012). Human topoisomerase I does not bind the substrate designed for REEAD and therefore cannot complete the circularization of the DNA strand.

Applying the bench-to-bedside-pathway to the REEAD method, product development and proof of principle have been completed in Denmark, leading to the next step of lab and field trials. As the test performance, which is the most important parameter to assess, is easily influenced in different settings, TDR recommends evaluating a test "*under the range of conditions in which they are likely to be used in practice" (Banoo, Bell et al. 2006).*

Regarding the new diagnostic method REEAD, this implies that the following step should be the systematic evaluation of the method in a malaria endemic setting.

4. Study purposes and objectives

The intended use of the index test, i. e. the microfluidic assay which is under evaluation, is for screening and diagnosis of malaria infections. The general purpose of this new method is to be able to test a large number of individuals in a patient-friendly, non-invasive manner using saliva as specimen. Thereby, improved surveillance and monitoring systems also for asymptomatic malaria infections could be set in place, since populations living in endemic areas could be screened for infections repeatedly in a more convenient way than it was possible ever before.

The overall objective of this diagnostic development programme is to develop a highly reliable malaria diagnostic test, meaning a test which is, compared to currently existing gold standard diagnostics, equal or superior in sensitivity and

specificity. This test should perform excellently in terms of its positive and negative predictive value and should have a very low parasite detection limit. Moreover, it ought to be working at a minimal technical complexity in order to be portable and usable in settings with very restricted resources and its use is desired to be intuitive also for people lacking any kind of specific training. In addition, it must be cost-effective and affordable for the target population it is designed for. This is the attempt to develop an easy to handle, point-of-care rapid diagnostic test processing saliva, which might be a future approach to tackle currently given obstacles in malaria control and could revolutionize malaria diagnostics.

The bench-to-bedside-pathway, which describes the different steps of the development of a new diagnostic test, is a multi-step process. The droplet microfluidics assay for malaria detection in human saliva has been developed in Denmark and transferred to Gabon, a malaria endemic country. Proven to operate smoothly in Denmark in the phase of proof-of-principle, different obstacles had to be faced once the method was performed in Gabon. Under the given circumstances, diverse adjustments of the assay had to be made before moving on to the next phase, where parameters such as sensitivity and specificity can be assessed in comparison to conventional malaria diagnostic methods.

The goal of the here described pilot study was to pave the way to further evaluations of the test in the field. Though initially developed and assessed in European laboratories, the methodology needed adaptation to the particular conditions of the implementation site, including variations in climatic factors such as heat and humidity, as well as constraints related to resource availability and shipment limitations. Optimal refinements would enable the execution of the assay by operators with minimal scientific or medical training in diverse settings, ranging from laboratories and healthcare facilities to potentially even patients' domestic environments. Therefore, the main objective was to optimise the droplet microfluidics assay and to advance the process of implementation in Gabon, the region where its future evaluation and use are planned. Specific study endpoints in this phase were to adjust the assay to the local setting in order to improve the

quality of results and to be able to reliably distinguish malaria positive from negative saliva samples, whose matching blood smear microscopy results were used as a reference test. Furthermore, a SOP was to be elaborated to document and standardize the laboratory procedures concerning the microfluidic assay.

In the available work, the process from technology transfer via optimisation towards the implementation of the method are being presented. Different adaptions are being described and explained and arising limitations are being explored and evaluated.

II Material and Methods

1. Study Region and Population

This work was conducted at the Centre de Recherches Médicales de Lambaréné, CERMEL (see Figure 4), in the central African country Gabon.

Gabon has a surface area of about 267600 km^2 and boundaries with Cameroon, Equatorial Guinea and the Republic of Congo and is located at the coast of the Atlantic Ocean. At the height of the equator running through Gabion, 81% of the countries surface is covered with tropical rainforest, resulting in hot and humid tropical climate. The year is divided by a short rainy season from October to December and a long rainy season from February to June with dry seasons in between. An average rainfall between 1500 and 3000mm per year is being reported (CIA 2024). Gabon is home to approximately 2.4 million people, of which about 91% is urban population and around a third of the total population is living in the capital city Libreville (Auswärtiges Amt 2021). Gabon has a very young population with a median age of 21 years and approximately 60% of the population being younger than 25 years old. The population growth rate is slightly above 2 and the fertility rate is 3.3 children per woman, indicating a high newborn and infant mortality. The official language is French, but more than 40 different Bantu-languages are spoken in Gabon (DGS 2013), (DGS 2022).

The study site Lambaréné is located in the inland at around 75km south of the equator in the province Moyen-Ogooué. It has about 24000 inhabitants and is surrounded by dense forests and the river Ogooué, whose two branches divide the town into an island and two river sides (Lim, Fernandes et al. 2021). Located at the river site, the history of the medical research center CERMEL in Lambaréné has its origins in the 1980s, where it was founded as part of the Albert-Schweitzer-Hospital with the purpose of studying the local populations diseases (Ramharter, Adegnika et al. 2007). Today, the research institution has an over 30-year long history of conducting clinical trials with focus on malaria, tuberculosis, HIV and other endemic parasitic diseases as well as performing

Figure 4 The laboratory building of CERMEL in 2017

Source: Author

basic immunological and molecular research. Nowadays, CERMEL is independently contributing to public health interests and is no longer an administrative part of the Albert Schweitzer-Hospital, but nevertheless both institutions continue to benefit from their proximity to each other. During the past years, its territory has been extended to a research campus and today, over 200 both national and international staff members are employed at CERMEL. Collaborations with various universities have been established and the institution has become an important training center for Gabonese as well as European students and medical doctors (Ramharter, Agnandji et al. 2021).

1.1 Malaria in the study region

According to Gabon's national demographic and health survey from 2012, malaria is still the first cause of morbidity among all groups of age (DGS 2013). Malaria transmission is perennial, meaning transmissions are occurring throughout the year, with highest disease burden during the rainy seasons, especially in October and November. Malaria vectors in Gabon are the female mosquitos of the *Anopheles funestus* and *Anopheles gambiae* species (Longo-Pendy, Boundenga et al. 2022). *Plasmodium falciparum* is the most prevalent malaria species, being responsible for more than 75% of all cases. Besides *P. falciparum*, *Plasmodium ovale wallikeri, P. ovale curtisi,* and *Plasmodium malariae* cause malaria infections in Gabon (Maghendji-Nzondo, Nzoughe et al. 2016). While in 2021 a total of around 141195 confirmed cases of malaria infections were reported countrywide, the estimated number of cases is as high as 535939 (WHO 2023a). A cross-sectional community survey of individuals of all ages that took place in February/March 2016 in the province of Ngounié, found a Plasmodium parasite prevalence of 37% with a range between 14% (1-2 years old) and 77% (9-10 years old) among different age groups (Manego, Mombo-Ngoma et al. 2017).

As stated by the national survey in 2012, every second Gabonese household owns a mosquito net, whether impregnated or not. 38% of the survey's participants declare to have slept under a mosquito net the previous night (DGS 2013). In the latest Gabonese national survey 2019-2021, a downward trend is described with only every fifth household owing an insecticide-treated mosquito net (DGS 2022). As indicated in the 2022 World malaria report, 13.8% of Gabon's population had access to an impregnated mosquito net in 2022 (WHO 2022). This stands in strong contrast to the WHO recommendation of at least one impregnated mosquito net per 2 people and according to WHO data, Gabon presents with the highest insecticide treated mosquito net ownership gap among the African Regions (WHO 2017).

Furthermore, it was found that among children under the age of 5 years, 24% have suffered from an episode of fever during the previous two weeks of the

survey (DGS 2022). Within these children, 67% were brought to seek health care and 15% of all feverish children received a blood draw for malaria diagnostics. A total of 26% of children with fever took antimalarial medication. These data show the discrepancy between appropriate diagnostics and targeted treatment of malaria. Malaria remains the most common cause of death among children younger than 5 years in Gabon (Countdown_to-2030 2015), (DGS 2013), (WHO 2016a), (WHO 2022).

1.2 Study design and facilities

In February 2017, the droplet microfluidic assay for malaria diagnostics in human saliva was transferred from Denmark to the research laboratory of CERMEL. The initial plan was to start a prospective observational, observer-blinded cohort study in the field within a few weeks after method transfer and implementation. A total number of 400 randomly collected saliva samples from individuals of all age groups from Lambaréné and Fougamou were planned to be analysed with the new assay and compared to blood smear microscopy and an RDT. For this study, ethics approval from the institutional ethics committee of CERMEL was obtained in February 2017 (internal reference: CEI-CERMEL 002/2017).
Since the method transfer posed several unexpected difficulties and in consequence, the method had to be first optimised in the new setting before the final implementation could take place, the study start had to be rescheduled and the pre-study project described in this work was initiated. This study was set out to systematically assess the technical performance of the assay and to optimise assay conditions to improve diagnostic performance of the assay.

To optimise the microfluidic assay, a library of samples and results had to be formed, which were available at CERMEL during the duration of this pilot-study. A number of 43 results of blood smear microscopy and RDTs, performed for malaria diagnostics for participants included in ongoing studies at CERMEL, were de-identified and anonymised and used as reference values in consultation with the participants original study responsible personnel. Complementary clinical and

demographical data (age, history of fever) were obtained in agreement with the original studies.

Patients of all ages presenting with fever (≥37.5°C axillary) or history of fever in the last 24 hours at the medical research centre in Lambaréné were screened for malaria infections, if no other cause for fever was known or obvious. These patients were informed about the study objectives and processes and eligible participants who gave their consent were asked to provide a saliva sample, which was collected in an unstimulated and spontaneous way into a 50ml cup with lid (CEB Qualibat, LOT 6056626) and immediately stored in a fridge with an average temperature of 4°C. In case of patients younger than 18 years old, a parent or legal guardian gave their consent, adolescents between 12 and 17 years of age additionally gave their assent for study participation. The provided sample was labelled in a de-identified and anonymous manner, so it could be matched with the blood test results, without revealing the patient's identity or origin. Since conventional malaria diagnostics were being performed in parallel for each patient, there was no necessity to provide the patients with the result of the experimental microfluidics assay. Since for the intended study a representative sample was aimed for, there were no exclusion criteria for the pre-study project. The microfluidic assay was performed on the same or the following day after sample collection, if not indicated differently.

All droplet microfluidic assays were performed in the research laboratory of CERMEL. The so-called "Labo des Recherches" consists of a main laboratory equipped with fridges and freezers, a main working bench (see Figure 5) and general supplies such as weighing scales, distilled water and other common reagents. In addition, more specific equipment is placed in the main room and enables analyses using a flow cytometer or performing ELISA. Furthermore, a cell culture room equipped with different flow hoods, incubators and centrifuges and moreover strictly separated pre- and post-PCR rooms with separate fridges, freezers and material stocks are part of the laboratory unit. Saliva samples were prepared, processed and incubated in the main laboratory and all reactions and steps described in this work took place using the main bench as shown on Figure

5. To avoid any contamination, solely the described Mastermix was prepared in the pre-PCR room and all its reagents and supplies were stored only in this dedicated room. A different laboratory coat and new gloves were worn in the pre-PCR room and were left in the room to further reduce the risk of contamination.

Figure 5 The main bench in the research laboratory of CERMEL in 2017

Source: Author

2. Conventional Procedures of Malaria Diagnostics

A blood sample was taken via a finger prick on the left index finger and a malaria rapid diagnostic test based on HRP-2 (ParaCheck Pf, LOT 311740) was being performed. The result of the RDT was reported as positive, when the control band and the test band became visible; the test was judged as providing a negative result when only the control band became visible and as invalid, when no band developed. In case of invalid test results, the test was repeated once.

Furthermore, two times a thick and a thin blood film each were prepared on two glass slides for microscopy diagnostics. For preparing thick films, a 10µl pipette

(CLINIPET, SN 82208721) was used to evenly spread 10µl of capillary blood over a rectangular area of 10x18mm, using a template placed under the slide.
Malaria microscopy slides were dried in a slide warmer (premiere XH-2004) and the thin films were fixed with 90% Methanol (MEDILAB; LOT 14020192). After that, all slides were stained with 20% Giemsa solution (Giemsa R solution, RAL diagnostics, LOT E19621; Buffer pH7,2, Merck KGaA chemicals, LOT TP1267368515) for 20 minutes. Following, the slides were rinsed with tap water and dried again. Certified microscopists were reading the stained slides according to the Lambaréné method. Therefore, depending on the number of parasites counted in the thick smear, 30 to 100 high power fields (HPF) were observed under a 100x objective (3 Nikon eclipse E200, SN 105126, SN 943687 and SN 145355; Olympus CH30F200 SN 7K07730) with immersion oil (DPX new, Merck KGaA chemicals, LOT HX60964379).
The parasitaemia was calculated in accordance with the following formula:

$$\frac{Parasites}{\mu l} = \frac{N(Parasites)}{N(HPF)} \times Microscope\ Factor$$

The microscope factor (MF) is individual for each microscope and represents the number of fields that have to be read in order to examine 1 µl of blood, depending on the microscope's magnification and area of one field. The MF can be calculated or measured physically.
According to CERMELs SOP on malaria slide reading, thick smears were read twice by two independent readers and results were compared afterwards. A third lecture was performed by a third independent person, if necessary, e.g. if the results of first and second reading differed more than 50% of the calculated parasitaemia or if the same slide was read positive and negative by two independent readers. The final parasitaemia was recorded as the average of the first two readings, or, if a third reading was performed, the average of the two closest results. The thin film on the slides was used for species identification, if necessary.

Patients with a positive microscopy result were treated with Coartem® (Artemether-Lumefantrine, NOVARTIS) according to their body weight (see Table 1)

Table 1 Dosing of Coartem®

Body weight	Dose of Coartem®
5-<15 kg	1 tablet of 20/120 Coartem® 2x/day for 3 days
15-<25kg	2 tablets of 20/120 Coartem® 2x/day for 3 days
25-<35kg	3 tablets of 20/120 Coartem® 2x/day for 3 days
≥35kg	1 tablet of 80/480 Coartem® 2x/day for 3 days

3. The droplet microfluidic assay for detection of malaria parasites in human saliva

The droplet microfluidic assay aims to detect malaria infections in human saliva. A hand driven enzyme extraction is being combined with a DNA amplification mechanism and followed by a colorimetric readout. In this chapter, the reagents, chemicals and equipment needed in order to carry out the microfluidic assay are listed and following, the principle as well as the use of controls and quality control are being explained.

3.1 Reagents and chemicals

- Binding buffer CP (Omega Biotek PDR042)
- BSA (bovine serum albumin)
- dH_20
- DNA substrate (5'-TCTAGAAAGTATAGGAACTTCGAACGACTCAGAATGACTGTGAAGATCGCTTATCCTCAATGCACATGTTTGGCTCCCATTCTGAGTCGTTCGAAGTTCCTATACTTT-3')
- dNTP (1mM of each of the nucleotides dATP, dCTP, dGTP and dTTP. 10% of dCTP was substituted with Biotin-16-Aminoallyl-2'-deoxycytidine-5'-Triphosphate from Trilink Biotechnologies)
- Drop release agent (1H,1H,2H,2H-Perflouro-1-octanol; Sigma 370533-25G)
- DTT (Dithiothreitol)
- EtOH 95%
- HRP (Pierce Streptavidin Poly-HR, 21140)
- Milk powder
- Oil (2% Pico-Surf (TM) 1 in Novec 7500; Dolomite- microfluidics, 3200215)
- PBS (Dulbecco's Phosphate Buffered Saline; Sigma, D8537)
- Phi29 buffer supplied with the Phi29 enzyme
- Phi29 polymerase (ThermoFisher Scientific, EP0094)
- PMSF (Henylmethylsulfonyl fluoride)
- Primer 1 (5'-CCT CAA TGC ACA TGT TTG GCT CC)
- Primer 2 (5'- CCA AAT AAG CGA TCT TCA CAG T)
- TE-buffer (Ethylenediaminetetraacetric acid; 10 mM Tris-HCl pH 7,5; 1 mM EDTA)
- TMB (TMB Enhanced One Component HRP Membrane Substrate; Surmodics, ESPM-0100-01)
- Tris-HCl
- Tween 20

  Urea powder

3.2 Equipment

- 200µl tubes
- 500µl tubes
- Blank paper
- DNA columns (Omega Biotek, DNACOL2-01)
- Eppendorf tubes 1.5ml
- Glue
- Inlet tubing (VWR, HAMI90624)
- Microfluid chip
- Outlet tubing (VWR, HAMI20924)
- P1000 pipette + tips (Thermo scientific, Finnpipette F2, SN: NH03817)
- P2 pipette + tips (Thermo scientific, Finnpipette F2, SN: NH04326)
- P20 pipette + tips (Thermo scientific, Finnpipette F2, SN: NH0446)
- P200 pipette + tips (Thermo scientific, Finnpipette F2, SN: NH03445)
- Paper scanner (CANON Maxity MB5050)
- Parafilm
- Scissors
- Syringes 1ml (Abena, 22801201)
- Table centrifuge (Galaxy minicentrifuge, VWR, SN 12040850)
- Transparent file
- Vortex mixer (neoLab, SN 07-2020)
- Incubator (water jacketed CO_2 incubator, 37°C, 0.3% CO_2; Thermo scientific, SN 308609_9150)

3.3 Principle

The topoisomerase I microfluidic assay consists of the four following major steps, which will be explained in this chapter:

1. Lysis of the malaria parasite and extraction of the target enzyme (pfTopoI)
2. Rolling circle amplification of the converted DNA substrate
3. Visualization of amplified DNA circles
4. Readout

Step 1: Lysis of the malaria parasite and extraction of the target enzyme

As described earlier, it has been shown that the whole Plasmodium parasite including its active enzymes can be found in human saliva. The aim of the first step is the hand-driven lysis of the parasite in order to extract the target enzyme, the topoisomerase I. Therefore, a single drop of saliva is diluted 50 times in PBS to homogenize the sample and mixed with lysis buffer, a hypotonic, low-salt solution. Then, the DNA substrate as well as oil are added. By vortexing the mixture for one minute, water-in-oil droplets are being produced. After that, the created droplets are fed via a syringe into a microchip with a serpentine channel between an inlet and an outlet as illustrated on Figure 6.

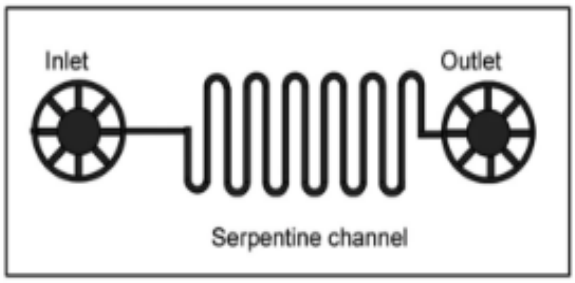


Figure 6 Illustration of the microfluidic chip for pump-free extraction of the pfTopoI from saliva from *Plasmodium* infected individual.
The microfluidic chip consists of an inlet and outlet site with a serpentine channel in-between. Droplets consisting of vortexed saliva, lysis buffer, oil and DNA substrate are loaded into the inlet side via a handheld syringe and collected via the outlet side.

Source: Hede, Fjelstrup et al. 2018

The mechanical stress that is being administered by pressing the emulsion through the chip causes the *Plasmodium* parasite to lyse, releasing the target enzyme. On the outlet side, the droplets are collected into a tube and incubated overnight. Once freed, the topoisomerase I is then able to convert a previously added hairpin-shaped DNA substrate into a covalently closed circular DNA due to its cleavage-ligation activity. As soon as the ligation is completed, the enzyme is released and ready to catalyse another substrate conversion process. The

process of the circular DNA creation by the topoisomerase I is illustrated on the upper part of Figure 7, depicted as step I-III.

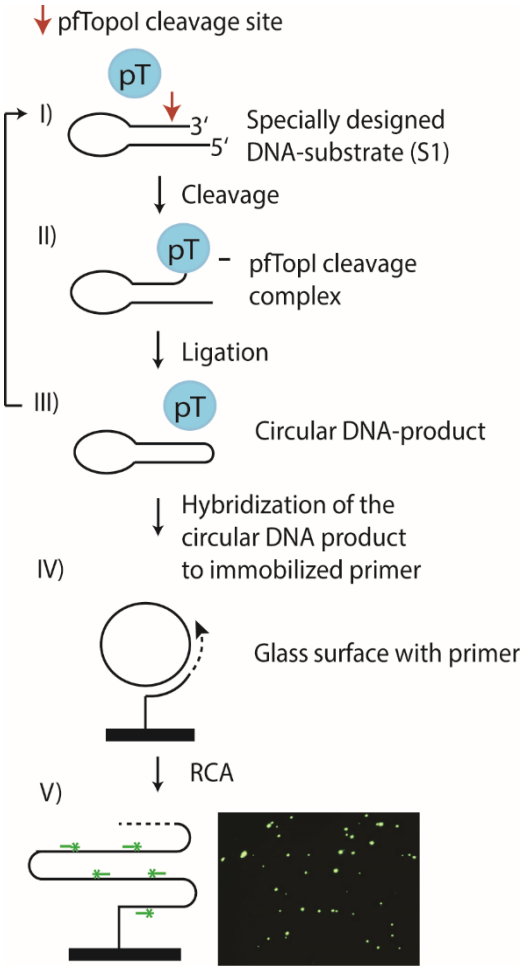


Figure 7 Schematic depiction of the REEAD assay

I) the DNA-substrate (S1) carrying a pTopI cleavage site (red arrow). II) pTopI (represented by a blue oval labeled pT) mediated cleavage leads to covalent attachment of the enzyme to the DNA-substrate. This covalent intermediate is denoted the cleavage complex. In a subsequent pTopI mediated ligation step, III), a covalently closed DNA circle is formed and pTopI is released from the DNA and therefore able to catalyze a new cleavage/ligation event. IV) The circular DNA product is hybridized to an immobilized RCA primer, which is elongated in a RCA reaction. V) Left panel: The tandem repeat RCA product is detected using fluorescent probes (green) and fluorescence microscopy. V) Right panel: A typical microscopic view obtained performing the REEAD assay.

Source: Hede, Fjelstrup et al. 2018

Step 2: Rolling circle amplification of the converted DNA substrate

After the incubation, when the reaction catalyzed by the topoisomerase is completed, the droplets are broken via a drop release agent. In the following step, the generated DNA circles are amplified via the *rolling circle amplification* reaction (RCA). To do so, a mastermix consisting of RCA-primers, the phi-29 DNA-polymerase and DNA nucleotides, of which 10% of the dCTP has been replaced by biotin-linked dCTP in a buffer medium is added to the circles created in step 1. The polymerase in the mastermix carries out the elongation of the primers according to the template of the closed DNA circle. This step is presented on the bottom part of Figure 7, pictured as step IV and V. The specific phi-29 polymerase used in the microfluidic assay has very unique characteristics. It was found that the phi-29 polymerase can perform a 10000-fold amplification of a circular DNA template within a few hours, whereas conventional polymerases used for RCA were limited to 50-fold amplifications. In addition, it consists of a single subunit, which operates stable at an optimum of 30°C for 12 hours and its proofreading ability ensures an average error rate of 1 in 10^6-10^7 bases. Furthermore, it can incorporate more than 70,000 nucleotides without disassociating from the DNA strand (Dean, Nelson et al. 2001).

Step 3: Visualization of the amplified DNA circles

After one hour of incubation at 37°C, the product is diluted 10-fold in TE buffer and hereafter 4-fold in CP buffer. Thereupon it is loaded on a DNA purification column and allowed to bind to the silica membrane during 10 minutes on a shaker at room temperature. Afterwards the whole volume is spun through the column. Next comes a series of washing steps, beginning with twice 200µl PBS, then a 10-minute blocking with 200µl blocking buffer and washing twice with 200µl PBS again. Following, the membrane is incubated with 200µl 8M Urea and again washed twice with 200µl PBS. Then 150µl Streptavidin-HRP in blocking buffer is added and incubated for another 10 minutes. Thereafter the column is washed three times with 150µl PBS supplemented with 0.2V% Triton X-100. The last step is performed by adding 150µl TMB to the columns. TMB is a substrate of HRP,

which catalyzes an oxidation reaction that converts the TMB into a blue product bound to the membrane. When the desired colour of the filters is reached, the reaction is stopped by spinning the column, adding 200µl distilled water and spinning again.

HPR, which stands for horseradish peroxidase, is an enzyme which is, as stated by its name, obtained from the root of the horseradish plant. The enzyme contains a heme-group and utilizes hydrogen peroxide to oxidise its substrate (Veitch 2004). As described above, HRP is given to the DNA columns, where it is coupled with biotin-linked nucleotides in the circular DNA end product, given that the saliva sample carries a current malaria infection and therefore the present topoisomerase has converted the DNA substrate into the mentioned end product. In the following step, TMB, so called Tetramethylbenzidine, is added. TMB, which is the most commonly used chromogenic substrate of HRP, is then oxidised by the HRP and hydrogen peroxide, resulting in a blue complex (Dorado, Okoth et al. 2016). The generation of the described complex is subsequently the colour reaction taking place on the filter, which can be observed in malaria infected samples during the last step of the assay. The visualisation of the DNA circles as converted by the topoisomere I enzyme present in *Plasmodium* parasites is shown on Figure 8.

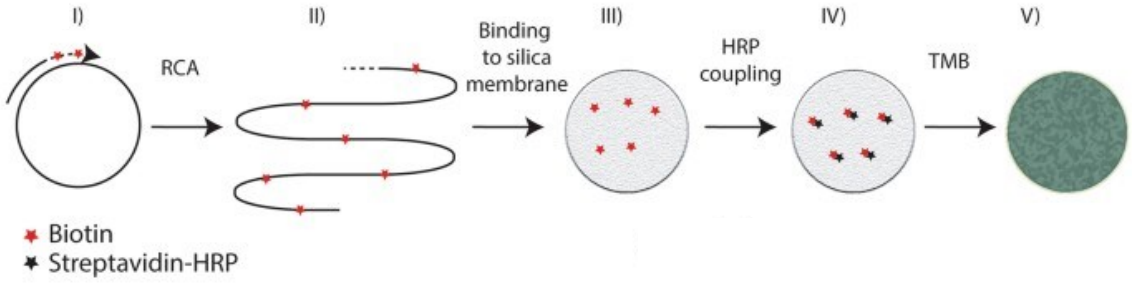


Figure 8 Detection of pTopI using a colorimetric readout
The individual steps of the colorimetric readout are schematically depicted. (I) shows the RCA primer hybridized to the pTopI generated single stranded DNA circles. II) The primer is elongated in a RCA reaction performed in the presence of biotin labelled nucleotides (red asterisks). III) The biotinylated DNA generated is bound to a silica membrane, which is positioned in a column. IV) Silica bound biotinylated DNA is visualized by coupling to streptavidin conjugated HRP (black asterisks) followed by V) incubation with the colorimetric HRP substrate, TMB.

Source: Hede, Fjelstrup et al. 2018

Step 4: Readout

Saliva samples from malaria patients are expected to result in blue membranes whereas saliva from uninfected individuals are expected to result in uncolored membranes. This effect is due to the presence of the topoisomerase I enzyme found in the Plasmodium parasite. The active enzyme in malaria infected individuals' saliva converts the added substrate, which is then amplified and contains biotin-linked nucleotides. These nucleotides containing biotin are coupled with HRP, which converts its substrate TMB into a blue end product. If no parasite is present in the saliva, the DNA substrate remains linear, no biotin-linked nucleotides are incorporated neither bound to the membrane. Therefore, the HRP is not bound on the filter and TMB is not converted into the blue product.

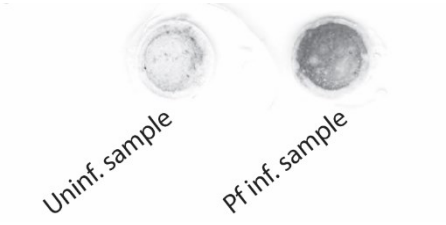


Figure 9 Comparison of an uninfected and infected patient sample on filters after completion of the assay.
The left sided filter of the sample an uninfected individual results in a clear filter, while the filter on the right side belonging to a *Plasmodium* infected individual turns blue.

Source: Hede, Fjelstrup et al. 2018

After the filters have been air dried, they can be removed from the DNA columns and scanned in a paper scanner in order to document the result of the microfluidic assay. Figure 9 demonstrates an example of two filters obtained after completion of the assay. The left-sided filter shows the analysis of an uninfected sample resulting in a blank filter. The right-sided filter illustrates the processing of a *Plasmodium* infected sample resulting in a coloured filter.

3.4 Use of controls and general quality control

Negative and positive controls were included in all individual experiments to ensure that the single steps of the procedure were working correctly and provided information for troubleshooting, respectively. Table 2 presents an overview of different types of controls as well as how they were generated and interpreted.

Table 2 Use of controls

Control type	Sample details	Explanation
Negative Control	True negative control saliva brought from Denmark	Demonstrating that there is no contamination of reagents or material and that background noise is minimal
Positive Control	Sample tested positive for malaria before (positive control 1)	Demonstrating that extraction of the parasite, amplification and readout of samples are done correctly
	Sample containing DNA substrate and the purified target enzyme pfTopoI (positive control 2)	Demonstrating that the DNA substrate is working
	Sample containing produced DNA circles (end product) (positive control 3)	Demonstrating that readout and amplification are working correctly

In each set of experiment, a negative control was processed via the microfluidic chip as described above before the first and after the last patient sample and handled as any other sample that was to be analysed. In step 2, for the rolling circle amplification, the positive control 3 was included for every experiment. Therefore, the mastermix was put to 2µl of premade DNA circles and following treated as the other samples. If required, i.e. when a new batch of substrate was used or in case of troubleshooting, positive control 2 was added. To do so, lysis buffer and oil were mixed with the purified pfTopoI enzyme in step 1. The mixture was not processed via the microfluidic chip, but vortexed and incubated overnight together with the remaining samples and then handled like any other sample to be analysed. Positive control 1, a sample tested positive in a previous experiment, was not applicable in this study phase.

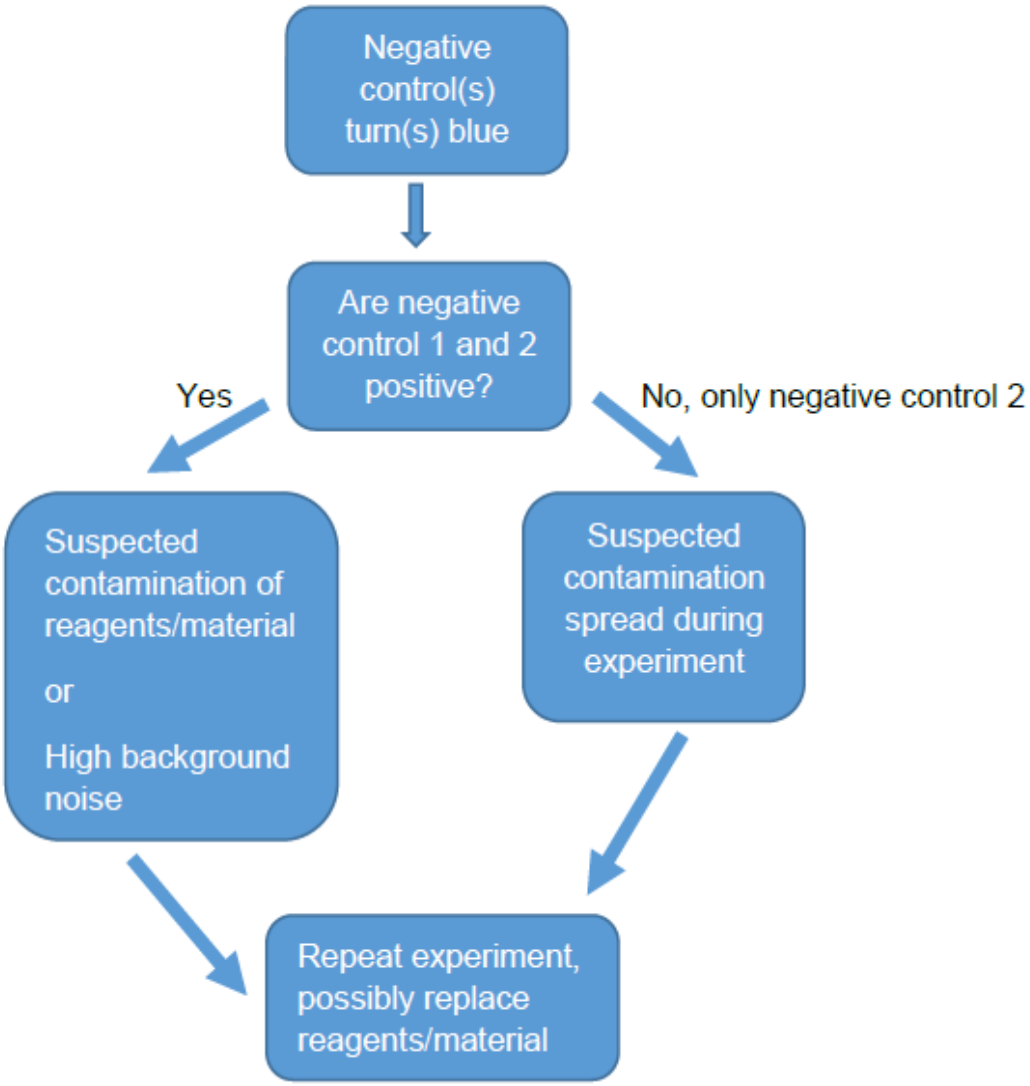


Figure 10 Scenario 1: Negative control(s) turn(s) blue
If both negative controls turn blue (left side), contamination of reagents and materials from previous experiments is suspected of general high background noise. In case only negative control 2 turns blue (right side), a spreading contamination within the experiment is assumed. In both cases the experiment has to be repeated.

Source: Author

Figure 10 depicts the analytical considerations undertaken during the readout process, specifically when the filter of a negative control exhibits a blue shade. The manifestation of positivity in both negative control 1 and negative control 2 raises suspicions of potential contamination in the reagents or materials.

Additionally, a heightened background signal on filters in a general sense may contribute to the phenomenon of negative control filters assuming a blue coloration. Conversely, if negative control 1 yields a blank filter while negative control 2 exhibits positivity, this observation prompts an inference of contamination occurring during the experimental procedure. In either scenario where negative controls exhibit characteristics of positive filters, it necessitates the repetition of the experiment.

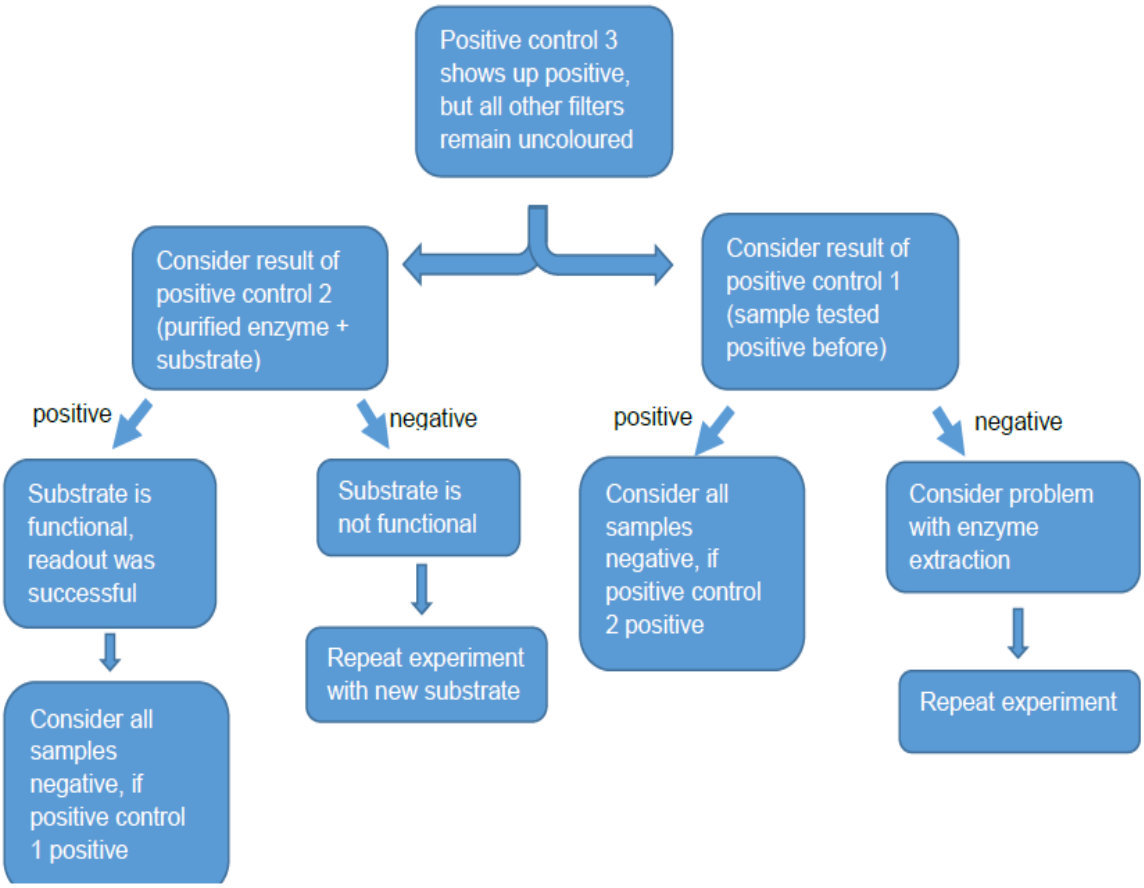


Figure 11 Scenario 2: only positive control 3 is positive
If positive control 1 and 2 are positive, all samples might be negative (left and middle right). If positive control 2 is negative, the substrate might not be functional (middle left). If positive control 1 is negative, enzyme extraction might not have been successful (right).

Source: Author

Figure 11 illustrates the subsequent considerations following the affirmation of only positive control 3, representing a sample containing the circular DNA as its end product. The left segment of the graphic depicts the outcome of positive control 2, derived from the DNA substrate and the isolated pfTopoI enzyme. Should positive control 2 yield positive results, indicating the functionality of the DNA substrate, and the readout step is operational, it is inferred that all samples may be negative, contingent upon the positivity of positive control 1. If positive control 3 is positive, yet positive control 2 yields a blank filter, suspicions arise regarding the substrate, necessitating the repetition of the experiment.
Conversely, the right segment emphasizes positive control 1, a sample that tested positively prior to the current experiment. If positive control 1 is positive, the likelihood of all samples being negative is dependent upon the positivity of positive control 2. However, if positive control 1 is negative while positive control 3 is positive, the occurrence of a complication during enzyme extraction is assumed, warranting the repetition of the experiment.

4. Filter presentation and quantification

The filters depicted in the figures presented subsequently were organized using FIJI (Image J picture processing software). Each filter was assigned a Region of Interest (ROI) measuring 0.6x0.6cm, and the filters were arranged in accordance with the processing sequence. The quantification of filters was also conducted utilizing the FIJI picture processing software. Throughout a single experimental setup, consistently circular ROIs (0.212cm^2) were specified, and the mean grey value was determined employing an inverted histogram (white = 255, black = 0).

Subsequently, all experimental counts per minute (cpm) values were normalized relative to the included positive control (100%, positive control cpm) and the mean value derived from both negative controls (0%, average negative control cpm).

Intensity [%] = ((experimental cpm – average negative control cpm) *100%) / (positive control cpm – average negative control cpm)

The brightness of the filters was gauged as x-fold enrichment, normalized against individual samples, negative controls, or blank filters, as indicated in the respective figures.

III Results

1. Study cohort and sample library

Between February and June 2017, samples from 43 participants were obtained to form a library of samples. A detailed list of characteristics of all analysed samples can be found in the Annex. Samples were collected from patients presenting themselves at CERMEL with fever, defined as axillary temperature ≥37.5°C, or history of fever in the previous 24 hours and no other known or obvious cause for fever.

Among these 43 patients who gave consent to give a saliva sample and have their age documented, the age ranged between 2 and 53 years with a median age of 7 years (Table 3). More than two thirds of the study cohort were children up to the age of 10 years old, 7% of study subjects were between 11 and 15 years old and 21% of all individuals were older than 15 years old (Figure 12).

Table 3 Patient age baseline values

Patient age baseline values	
N	43
Median	7
Range	2 – 53
Percentile 25 75	 5.0 11.0

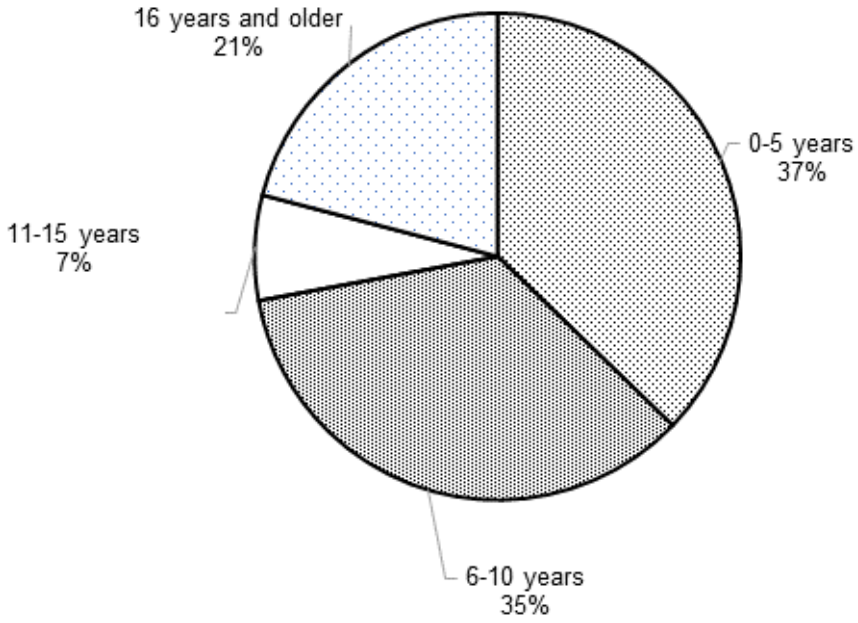


Figure 12 72% of the study cohort was 10 years and younger.
Percentual representation of the age groups 0-5 years (n=16), 6-10 years (n=15), 11-15 years (n=3) and 16 years and older (n=9) of the study cohort of 43 patient samples.

Table 4 Patient parasitaemia baseline values

Patient parasitaemia baseline values as microscopically diagnosed	
N	43
Median	753
Range	0 – 188100
Percentile 25 75	 0.0 3791.0

The recorded parasitaemia varied between 0 and 188100 parasites/µl peripheral blood, counted in the thick smear microscopy with the Lambaréné method. The median parasitaemia was 753 parasites/µl peripheral blood (Table 4). 32% of

study participants had a negative thick smear in microscopy. Among individuals with positive microscopy results, 14% had between 1 and 500 parasites/ µl, 9% presented with 501-1000 parasites/µl, the largest group of 26% exhibited a parasitaemia falling within the range of 1001-5000 parasites/µl and 7% showed between 5001 and 10000 parasites/ µl. Furthermore, it is noteworthy that roughly one in ten participants (12%) in the study manifested a parasitaemia exceeding 10001 parasites/µl, as illustrated in Figure 13.

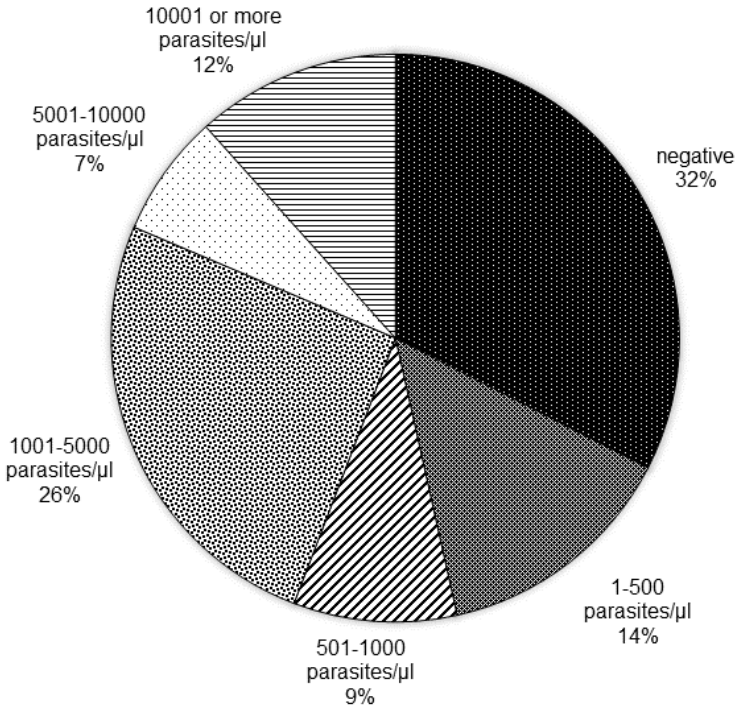


Figure 13 Most patients with parasitemia belonged to category 4 with 1001-5000 parasites/µl.
Percentual representation of different degrees of parasitemia (Category 1-6) in the study cohort of n=43 patient samples.

In the analysis of median parasite counts across different age cohorts, a discernible trend emerged wherein parasite burden exhibited a decrease with advancing age (Figure 14). Notably, children within the age bracket of 0 to 5 years manifested the highest median parasitaemia with a range of 1001-5000 parasites per microliter. Subsequently, individuals aged 6 to 15 years displayed a median

parasitaemia falling within the range of 501-1000 parasites/µl. Remarkably, participants aged 16 years and above exhibited a higher propensity for negative results in blood smear microscopy. Elevated levels of parasitaemia exceeding 10,000 parasites/µl were observed across all age groups, except in the case of teenagers between 11 and 15 years old. It is pertinent to note that the latter age cohort comprised only three participants within the study.

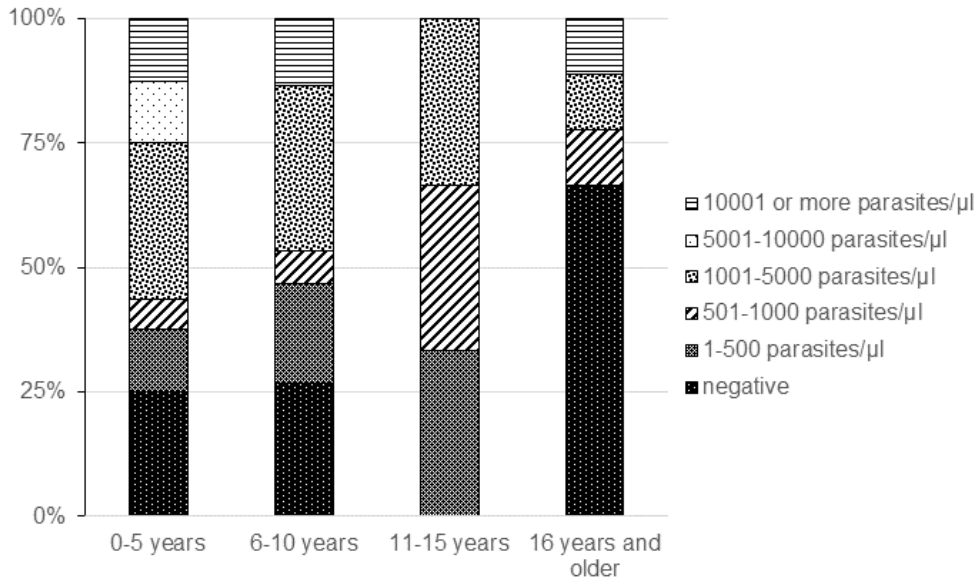


Figure 14 Degrees of parasitemia vary per age group of the study cohort.
Percentual representation of different degrees of parasitemia (Category 1-6) per age groups of 0-5 years (n=16), 6-10 years (n=15), 11-15 years (n=3) and 16 years and older (n=9). Category 1 defined as negative, category 2: 1-500 parasites/ µl, category 3: 501-1000 parasites/ µl, category 4: 1001-5000 parasites/ µl, category 5: 5001-10000 parasites/ µl, category 6: 10001 and more parasites/µl.

As depicted on table 5,out of the 43 performed rapid diagnostic tests, 36 were positive for Plasmodium falciparum while 7 tests were negative. Regarding the microscopy results, in 29 blood smears Plasmodium parasites were present while 14 blood smears were read malaria negative. Consequently, within the 43 performed rapid diagnostic tests for malaria, 7 (16.3%) were false positive results, meaning that there were no parasites detectable by microscopy.

Table 5 Results of thick smear microscopy and RDTs among study population

	RDT positive	RDT negative	Total
Thick smear positive	29 (67.4%)	0 (0.0%)	29 (67.4%)
Thick smear negative	7 (16.3%)	7 (16.3%)	14 (32.6%)
Total	36 (83.7%)	7 (16.3%)	43 (100%)

Among the 43 collected samples, gametocytes were microscopically detected in one thick blood smear (7 gametocytes per microliter blood). One mixed malaria infection consisting of *Plasmodium falciparum* and *Plasmodium ovale* was microscopically detected.

With the available 43 saliva samples, a total of 23 sets of experiments have been performed, of which several were carried out in multiple readouts. Per set of experiment, between one and ten samples were analysed and numerous saliva samples were analysed more than once in different sets of experiments. If strongly positive samples were required for optimisation purposes, but available samples were either too old or not sufficiently parasitized, samples were boosted by adding DNA control circles (positive control 3) to the saliva sample in step 1, before pressing the sample through the microchip. Following, these samples will be labelled as “boosted”.

To arrange the scanned filters in the hereafter presented figures, a circular ROI was defined and kept constant for a series of filters. By measuring the mean gray values with inverted histograms for individual experiments, the color intensity of filters was determined and normalized against positive and negative controls. To determine the brightness of filters, fold enrichment over individual samples, negative controls, or original filters was measured. The microscopically determined parasitemia of blood samples was correlated with the color intensity of the filters from the corresponding saliva samples.

2. Results of the first droplet microfluidics assay in Gabon

In Figure 15A the removed and scanned filters, on which the DNA has bound, are presented after the completion of all steps of the assay. These were the results of the first microfluidic assay performed in the laboratory of CERMEL with saliva samples from individuals from Lambaréné and surroundings, which had all positive and negative required controls included. Four individual samples from four different patients were taken and tested in the microfluidic assay (Fig. 15 A-E). The results were compared to the level of parasitaemia measured with the standard procedure of microscopical counting of parasites in a stained blood smear (Fig. 15D,E). Sample 2, 3 and 4, corresponding to 2166 parasites per microliter blood [p/µl], 1350p/µl and 4789p/µl respectively, were not enriched over the average intensity of the negative controls (Fig. 15B). However, sample 5, corresponding to 6869p/µl, was 1.53-fold enriched over the average of the negative controls. Overall, the correlation between filter intensity normalized to negative and positive controls and measured parasitaemia was significant ($p=0.0151$) with a linear regression with a fit of $r^2=0.9701$ (Fig. 15D). Figure 15E shows the correlation of individual samples between filter intensity and blood smear count parasiteamia for all four analysed samples in this assay. Thus, when looking at the individual patient samples, most apparent in Figure 15C, sample 2-4 were identified as negative based on the droplet microfluidic assay. Only sample 5 with the highest parasite count shows a filter intensity of 83% compared to the positive control.

Therefore, the first microfluidic filter assay performed in Gabon revealed a certain degree of unreliability at lower parasite counts. In addition, during the course of the procedure the negative control 2 exhibited a higher staining intensity than the negative control 1 that was performed as first sample. Through averaging the negative control intensities, we normalized to a median value for the patient samples. Nonetheless, the assay did not perform as stable during the course of the staining procedure, improving the reliability of the controls was objective of the next experiments.

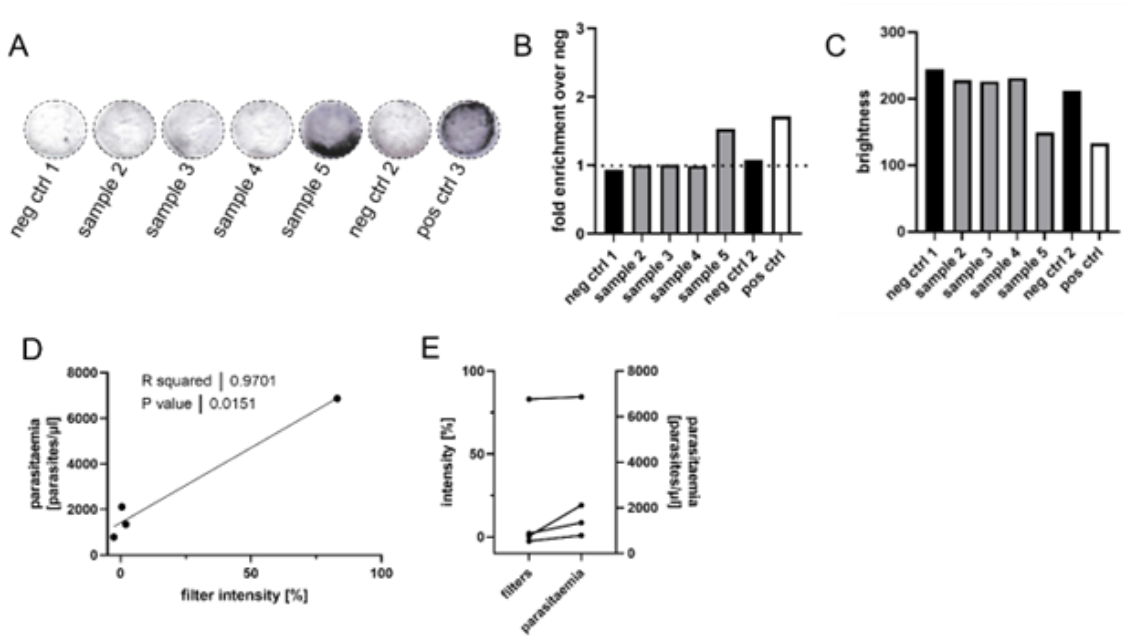


Figure 15 First droplet microfluidics filter readout performed in Gabon mostly correlated to microscopically observed degree of parasitemia, but failed to identify lower degrees of parasitemia.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample number 2 to 5, neg ctrl 2, positive control 3 (pos ctrl 3). Dark staining is indicating the presence of parasites.
B) Filter intensity measured as brightness as enrichment over the average of neg ctrl is plotted.
C) Measurement of filter brightness.
D)Linear regression analysis with R^2=0.9701 and p-value of 0.0151
E) Filter intensity in percentage of positivity (normalized to neg and pos ctrl) is correlated to the microscopically measured parasitemia by standard thick blood smear (number of parasites per µl of blood [parasites/ µl]).
N=1, 4 individual samples

3. Reducing contamination and background on negative samples

After the first complete assay was performed in Gabon and the limitations regarding the readout described above were identified, we focused on the high background of the negative control as a first step. It was remarkable that the second negative control resulted in a much darker filter than the first negative

control despite the same sample has been processed twice and result in a very similar colour on the filter. We suspected a contamination occurring during sample processing and considered that active parasitic enzymes from previous positive samples might have been spread on the following sample during the extraction step. This assumption could explain why the first negative control presents a much clearer filter than the second one. In Denmark the microfluidic chip and syringe were rinsed with oil and the tubings were reused without further washing, therefore the material was identified as possible source of contamination.

In order to achieve clearer filters of negative controls, different experiments were designed. An overview of the systematic approaches focusing on the colour of the negative control, which will be shown in detail in the following chapter, is presented in Figure 16. In a first approach, more washing steps with different reagents were gradually included. Thereafter, one after another material was replaced by single-use items. Bearing in mind that resources and access to material and reagents is limited in Gabon and shipping supplies from Europe is a time demanding and logistically complex process, an escalating course of action was chosen.

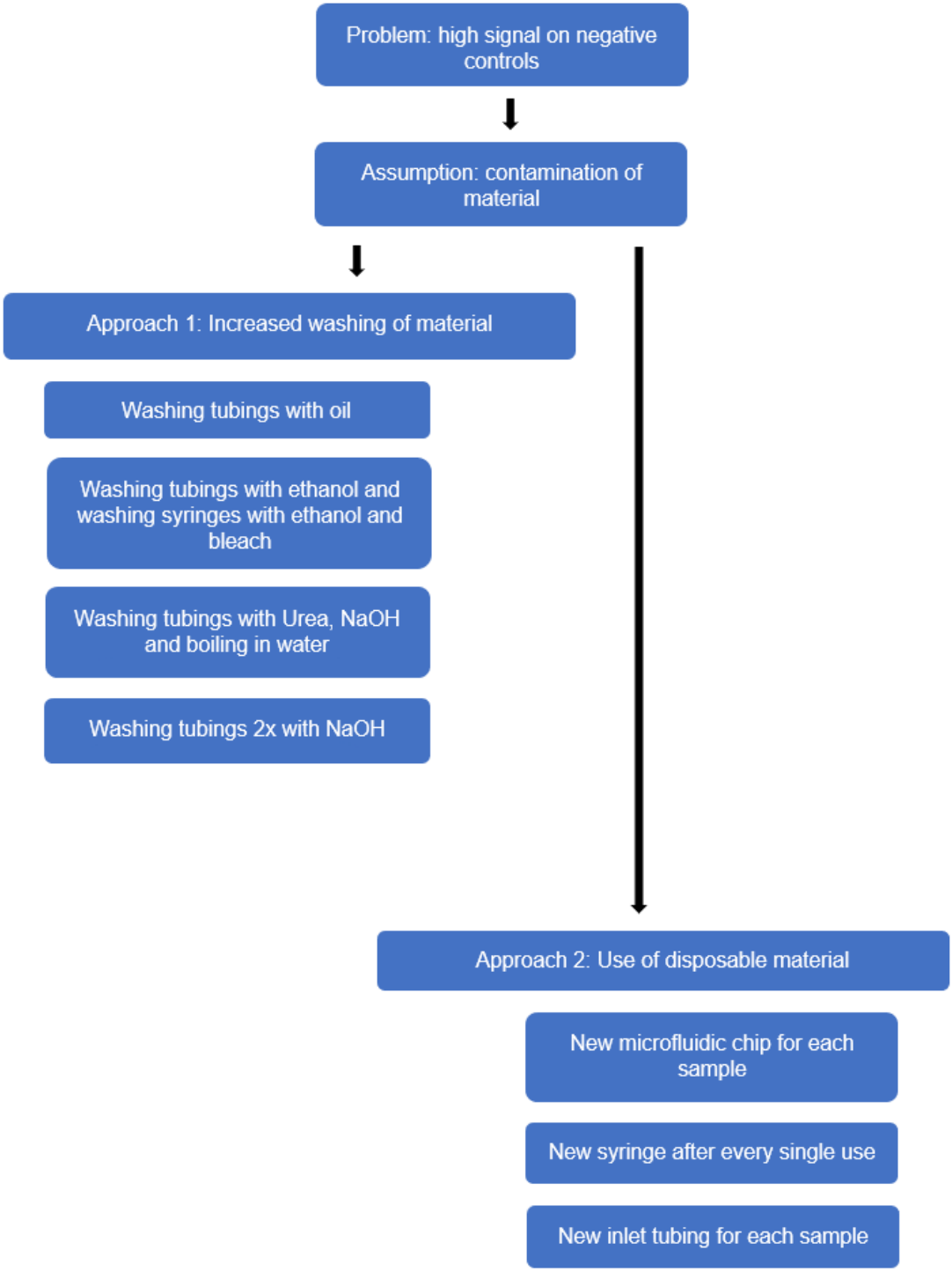


Figure 16 Visualisation of the two approaches to reduce high background negative filters.
Approach 1 was to examine increased washing of material (left side) and approach 2 to explore the use of disposable materials (right side)

3.1 Increased washing of material

A washing step was included to clean the tubing from the in- and outside with oil between sample processing in order to reduce contamination. A blank filter was used for better comparison of the staining of the negative control (Fig. 17A, C, D). The additional washing step did not bring any improvement as the negative control is as much coloured as the secondly processed positive sample (Fig. 17A, B, D). When normalized to the blank filter (Fig. 17C), sample 5 and the negative control were positive indicating a high background staining. Since washing with oil was not sufficient, we decided to include more stringent washing steps to decrease background signals.

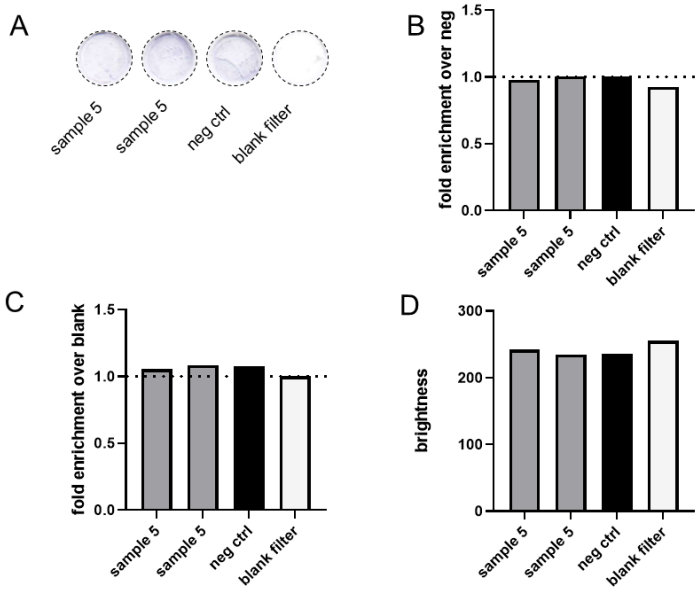


Figure 17 Increased washing of the tubings with oil did not improve the staining resolution.
A) Filter staining of droplet microfluidic assay of (left to right) sample 5 in duplicate, neg ctrl, blank, blank filter. Dark staining is indicating the presence of parasites.
B) Fold enrichment over the staining of the neg ctrl, measured as brightness, dotted line indicates value of neg ctrl set to 1.
C) Fold enrichment over the blank filter, measured as brightness, dotted line indicates value of blank filter measurement set to 1.
D) Measurement of filter brightness.
N=1

In Figure 18 the results after the addition of more aggressive washing steps are shown. The tubings were washed ethanol and the syringes, which are used to feed the emulsion into the microchip, were rinsed with ethanol and bleach. Again,

a blank filter was included for better differentiation (Fig. 18A-D). The negative control, which was processed last, shows the highest colour intensity (Fig. 18A-D). As the colour intensity increased according to the order of processing (Fig. 18D), we suspected an accumulative contamination, meaning that the enzymes or whichever agent is causing the negative samples to become positive sumed up with every sample that is being processed. When normalized over the blank filter, the increasing signal in accordance with the processing sequence and the remarkably high signal of negative control 2 can be observed (Fig. 18C). Moreover, positive sample 5 with 6869p/µl in corresponding blood smear microscopy had the same brightness as the blank filter when processed the first time (Fig. 18D), showing the overall weak signal intensity. To avoid contamination, we compared further washing methods.

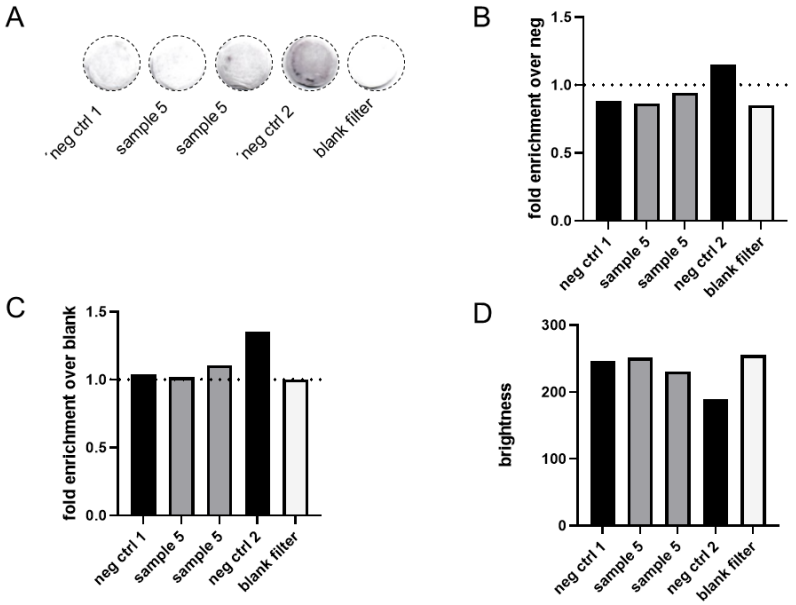


Figure 18 Increased washing of the tubings with ethanol and the syringes with ethanol and bleach did not result in decrease background staining of the negative control 2.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample 5 in duplicate, neg ctrl 2, blank, unstained filter.
B) Fold enrichment over the staining of the average value of the negative controls 1 and 2, measured as brightness, dotted line indicates value of average neg ctrl set to 1.
C) Fold enrichment over the blank filter, measured as brightness, dotted line indicates value of blank filter measurement set to 1.
D) Measurement of filter brightness.
N=1

Three different washing procedures for the inlet tubing, which is connecting the syringe with the microchip, were compared. The tube was washed with 6M Urea with 10M NaOH and as a third method it was boiled in water for two minutes. A negative control, a boosted positive sample and the second negative control were processed for each washing procedure (Fig. 19A). When normalized over the first and second negative control (Fig. 19B) washing the inlet tubing with NaOH looked most promising as both negative controls resulted in similar enrichment. Washing with NaOH was also yielding the most favourable signal-to-noise-ratio (Fig. 19).

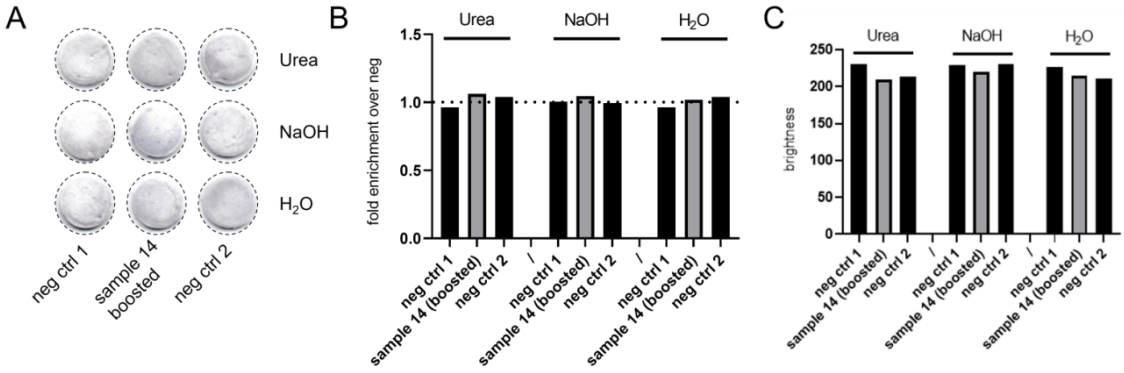


Figure 19 Increased washing of the tubings with NaOH decreased background staining of the negative control 2 slightly.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample 14 boosted, neg ctrl 2, washed in the first row with urea, second row NaOH and third row with H2O.
B) Comparative analysis of fold enrichment over the staining of the average value of the negative controls 1 and 2, measured as brightness, dotted line indicates value of average neg ctrl set to 1 per washing procedure.
C) Measurement of filter brightness.
N=1

In order to retest the washing with 10M NaOH, the experiment was repeated three times (Fig. 20A). To be sure that contamination after very strong positive samples could be avoided as well, the positive saliva sample was in addition boosted with the double amount of DNA circles as before (8µl instead of 4µl). Even after processing a strongly parasitised sample (in this case mimicked by a boosted sample), washing the inlet tubing with NaOH seemed to reduce contamination

resulting in two almost identically coloured negative controls in all three repetitions (Fig. 20A). Figure 20B shows a 1.27-fold enrichment of boosted sample 14 over negative for wash 1, 1.23-fold and 1.32-fold respectively for wash 2 and 3 and allowed a clear distinction between positive sample and negative control, also represented in the measurement of filter brightness (Fig. 20C).

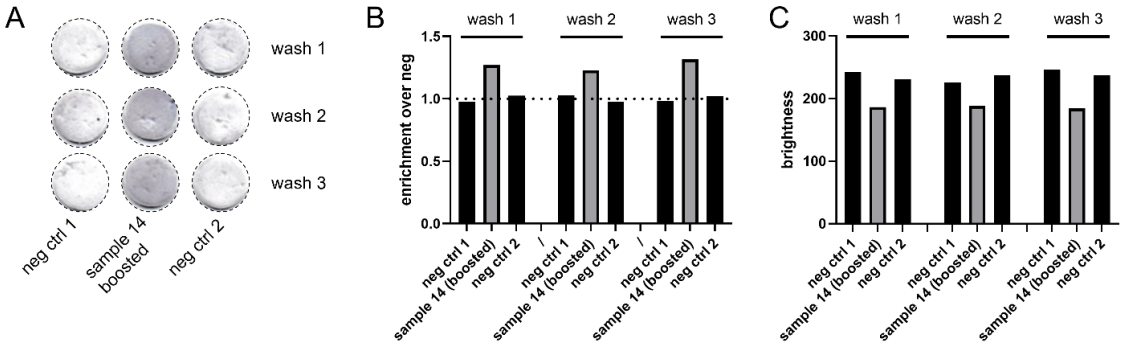


Figure 20 Increased washing of the tubings with NaOH resulted in reduced staining of negative controls.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample 14 boosted, neg ctrl 2.
B) Comparative analysis of fold enrichment over the staining of the average value of the negative controls 1 and 2, measured as brightness, dotted line indicates value of average neg ctrl set to 1 per washing procedure.
C) Measurement of filter brightness.
N=3

In three more repetitions, we aimed to further optimise the washing method (Fig. 21A). The reagent used to rinse the inlet tube and the syringe remained NaOH, but in two steps and in addition, the syringes were changed between the washing steps. Like in the previous experiment, first and second negative control resulted in almost identical colours (Fig. 21A-C). The greatest enrichment over negative control was reached in the first analysis (1.31-fold, Fig. 21B), which is similar to results of the preceding experiment (Fig. 20B). The difference in brightness between positive sample and negative controls (Fig. 21C) was less distinct. We assumed that only using new syringes and double washing of tubings with NaOH does not bring much advantage and therefore decided it was inevitable to assess the use of disposable consumables.

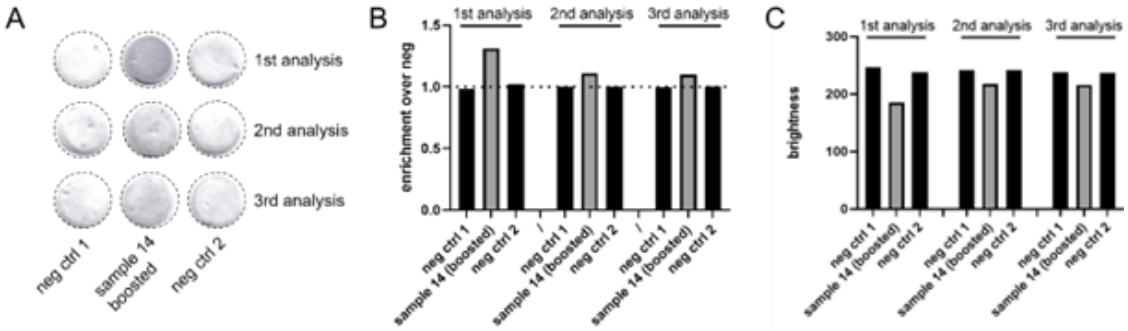


Figure 21 Double washing with NaOH and additional change of syringes did not further reduce the background staining of the negative controls.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample 14 boosted, neg ctrl 2, washed with NaOH and exchanged syringes between washes.
B) Comparative analysis of fold enrichment over the staining of the average value of the negative controls 1 and 2, measured as brightness, dotted line indicates value of average neg ctrl set to 1 per washing procedure.
C) Measurement of filter brightness.
N=3

3.2 Use of disposable material

Another approach to reduce and avoid contamination was to replace reusable by disposable material. Firstly, instead of using only one microfluidic chip per set of experiment and rinsing it with oil between the individual samples, a new microfluidic chip for each sample was used and in addition the syringes were discarded after a single use (Fig. 22A-C). The positive sample 5 could be clearly distinguished from negative control 1 and 2 (Fig. 20A). Furthermore, the negative control 1 which was processed first and negative control 2 that was processed last result showed identical brightness (Fig. 20C), indicating less contamination on subsequent samples. When normalized above the negative controls, the enrichment of the parasitised sample 5 became obvious (Fig. 20B). However, sample 5, which was analysed twice, did not develop identical signal intensities on the two filters.

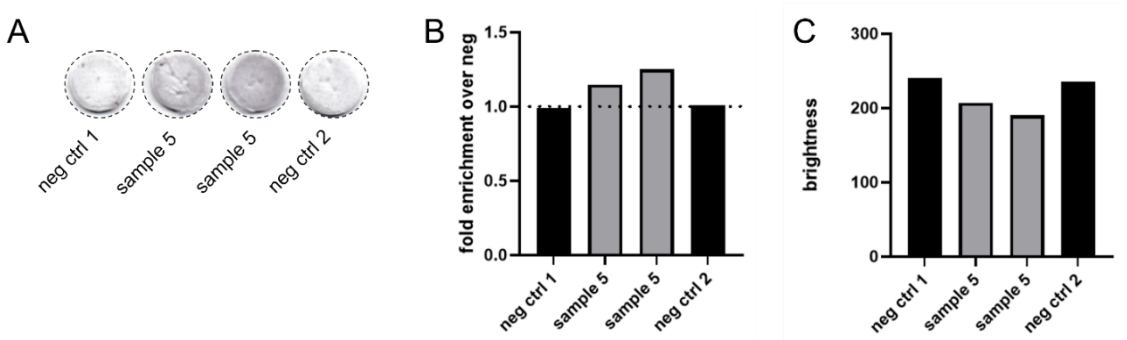


Figure 22 Replacement of microfluidic chip and syringe after single use increased staining resolution.
A) Filter staining of droplet microfluidic assay of (left to right) negative control (neg ctrl) 1, sample 5 in duplicate, neg ctrl 2. Dark staining is indicating the presence of parasites.
B) Fold enrichment over the staining of the averaged neg ctrl, measured as brightness, dotted line indicates value of neg ctrl average set to 1.
C) Measurement of filter brightness.
N=1

Furthermore, the use of the inlet tube was altered. Instead of washing the tube between processing the samples, now one inlet tube per sample was used (Fig. 23A-D). Overall signal intensity was weak for all samples except the positive control (Fig. 23A). In spite of that, the colour intensity of the positive control depicted that in general the assay is capable of generating intensely stained filters with almost 3-fold enrichment over negative (Fig. 23B). The correlation between filter staining and thick smear microscopy in this experiment was weak (R2=0.314, p-value of 0.092) (Fig. 23C) for both high and moderate parasitaemia (e.g. sample 25 88100 p/µl, sample 32 9113 p/µl) and also negative samples (e.g. sample 23,26,30) did not represent in equal filter intensities (Fig. 23D).

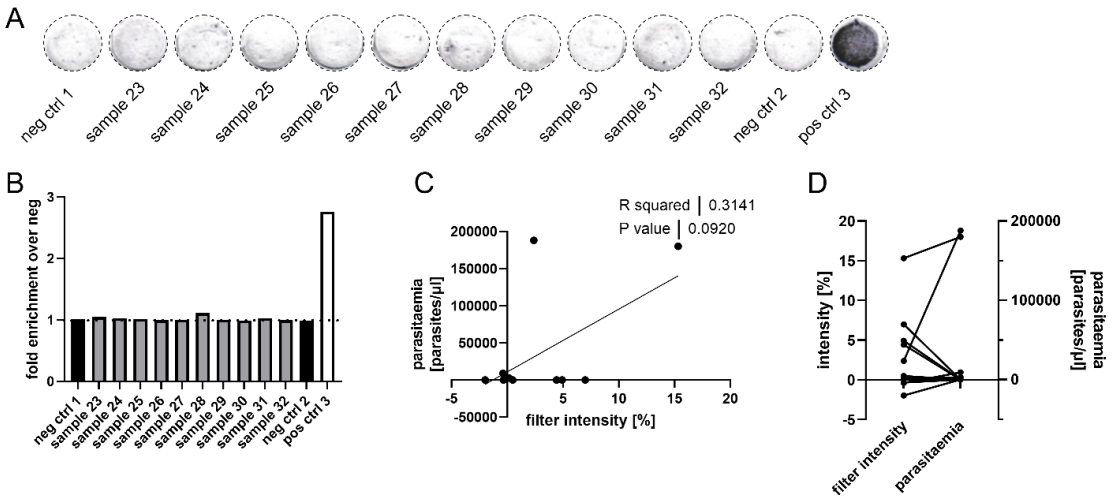


Figure 23 The usage of single use inlet tubing did not result in a robust correlation between filter staining and microscopically observed parasitemia.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample number 23 to 32, neg ctrl 2, positive control 3 (pos ctrl 3).
B) Filter staining measured as brightness is plotted as fold enrichment over the average of neg ctrls.
C-D) Filter intensity in percentage of positivity (normalized to neg and pos ctrl) is correlated to the microscopically measured parasitemia by standard thick blood smear procedure (number of parasites per µl of blood [parasites/ µl]).
C) Linear regression analysis with an R^2=0.314 and p-value of 0.092.
D) Correlation of individual patient values of filter intensity and microscopically measured parasites.
N=1, 10 individual samples

4. Enhancing the signals of positive samples

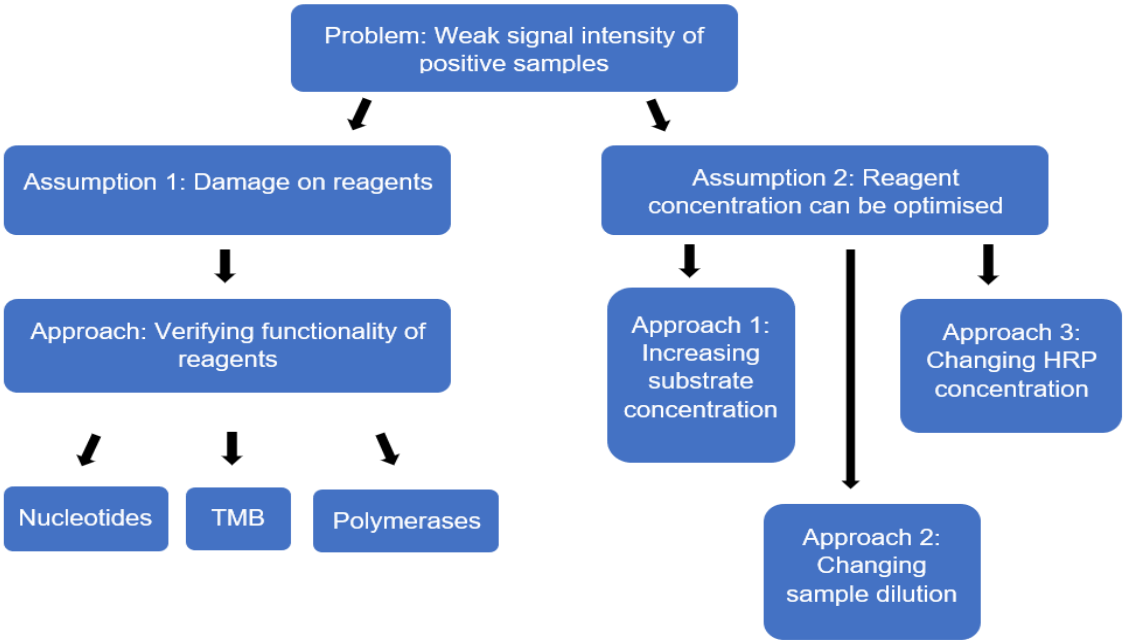


Figure 24 Graphic representation of the systematic analysis of weak filter intensity. Compromised reagent quality after shipment was assumed and the functionality of nucleotides, TMB and polymerases was evaluated (left side of graph). Optimised concentrations of substrate, HRP and sample dilutions were assessed (right side of graph).

The second obstacle that occurred was the weak signal on filters of positive samples. Two possible reasons were identified, firstly the damage on reagents during transport and storage and secondly the reagent concentration used to perform the assay. Figure 24 shows an overview of the different approaches that were further examined. To verify the functionality of reagents, TMB and nucleotides were re-tested in Denmark and the activity of the polymerase was analysed in Gabon. In order to determine the optimal amount of substrate concentration and HRP, different concentrations of the reagents were compared. Furthermore, the dilution factor of saliva samples was altered to find out if higher signal intensities can be reached. In the following chapter the results of these experiments are presented.

4.1 Verification of functionality of reagents

An aliquot of the TMB that was shipped from Denmark to Gabon was taken back to Denmark in order to retest the same samples with same TMB. This procedure allowed a comparison of results using the TMB that stayed in Denmark with results obtained with the TMB used in Gabon. The experiment conducted in Denmark using the TMB that was brought back from Gabon to Denmark as well as saliva samples collected in Gabon showed the desired colour intensity of filter membranes.

Furthermore, the status of the nucleotides used in Gabon was tested as they had been transported from Denmark to Gabon at -80°C. In order to examine the effect of low temperature on the DNA elements, nucleotides were exposed to -80°C in Denmark and then used to perform the microfluidic assay. Again, the experiment carried out in Denmark resulted in desired colour intensity and uncoloured filters of negative controls.

We assumed that the TMB used in Gabon should be fully functional and the low temperatures of -80°C the nucleotides were exposed to during transport should not have any impact on their integrity.

4.2 Increasing the substrate concentration

Different substrate concentrations were compared for negative and positive samples. The DNA substrate is a hairpin shaped single stranded DNA, which is converted into a closed circular DNA ring by the active pfTopoisomerase I found in all *Plasmodium* species. The substrate is given to the sample and incubated so the transformation can take place in infected saliva samples with the enzyme available. During the read out, only amplified circular DNA is made visible and results in coloured filters. The assay was performed with three negative samples, represented by negative controls in order to have real negatives, followed by three times a positive sample and last a second negative control (Fig. 25A). The applied substrate concentrations were the initially used concentration of 1µl substrate per 400µl lysis buffer (indicated as 1x substrate concentration) besides four times and eight times the initial concentration. When normalized over the first

and last negative control with initial substrate concentration, a considerable enrichment of all positive samples increasing with rising substrate concentrations was observed (Fig. 25B). However, higher substrate concentrations led to increased background staining on negative controls as measured in brightness of filters (Fig. 25C), hindering the discrimination of positive and negative samples. Therefore, the initially used substrate concentration was adopted in following assays.

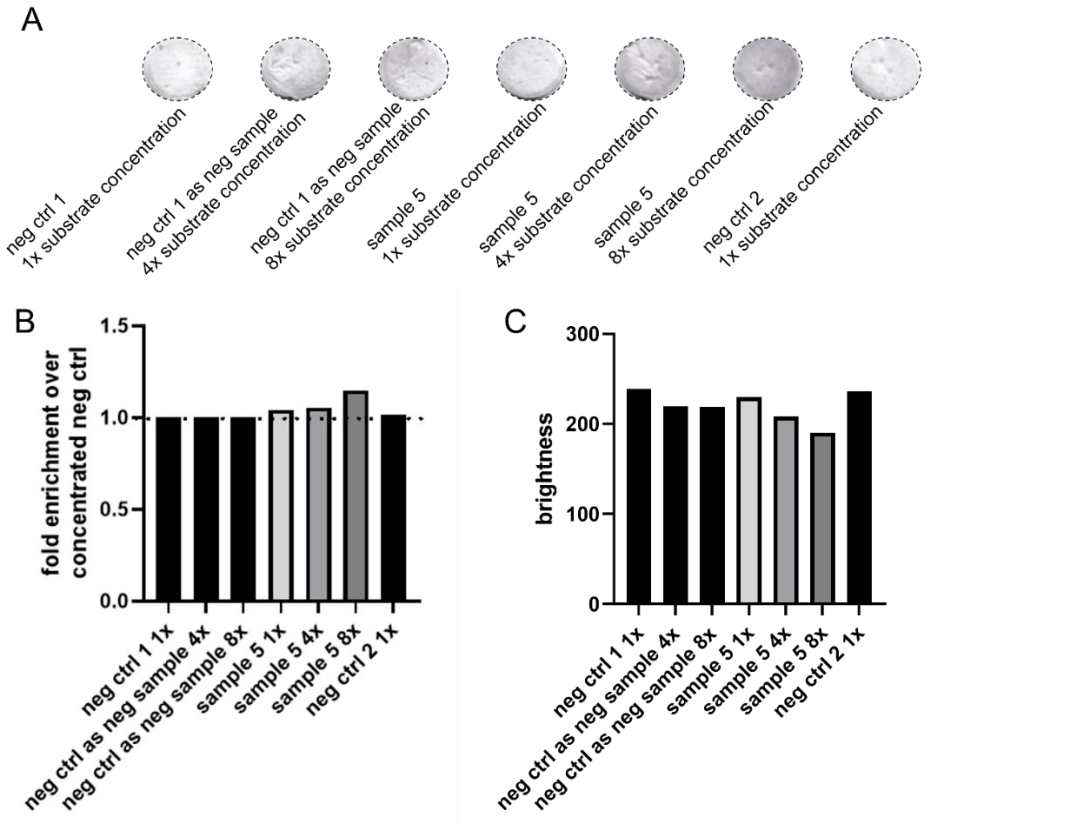


Figure 25 Increased substrate concentrations led to enhanced sample staining with higher negative background.
A) Filter staining of droplet microfluidic assay of (left to right) negative control (neg ctrl) 1 with initial substrate concentration 1x, four times concentrated 4x and eight times concentrated 8x, sample 5 in 1x, 4x and 8x concentrated and neg ctrl 2 not concentrated.
B) Fold enrichment over the staining of the corresponding concentration of neg ctrl, measured as brightness, dotted line indicates value of neg ctrl per concentration set to 1.
C) Measurement of filter brightness.
N=1

4.3 Testing different polymerases against each other

Two identical stocks of phi-29 polymerases were brought from Denmark to Gabon, Polymerase 1 was transported and upon arrival stored at -20°C, Polymerase 2 was transported and afterwards stored at 5°C. In order to rule out a possible impact of temperature on their functionality, the two stocks of polymerases were compared. To additionally test the polymerases' level of activity, different concentrations of DNA control circles were analysed. Therefore, DNA control circles were used undiluted, in a 1:1 dilution with TE and in a 1:3 dilution with TE (Fig. 26A). As expected, higher concentrations of control circles resulted in gradually higher colour intensities on the filters since more rings can be amplified by the polymerase in the given time frame during RCA, leading to a higher colour intensity during the read-out step (Fig. 25B). From the obtained results we assumed that none of the polymerases was harmed during transport and storage.

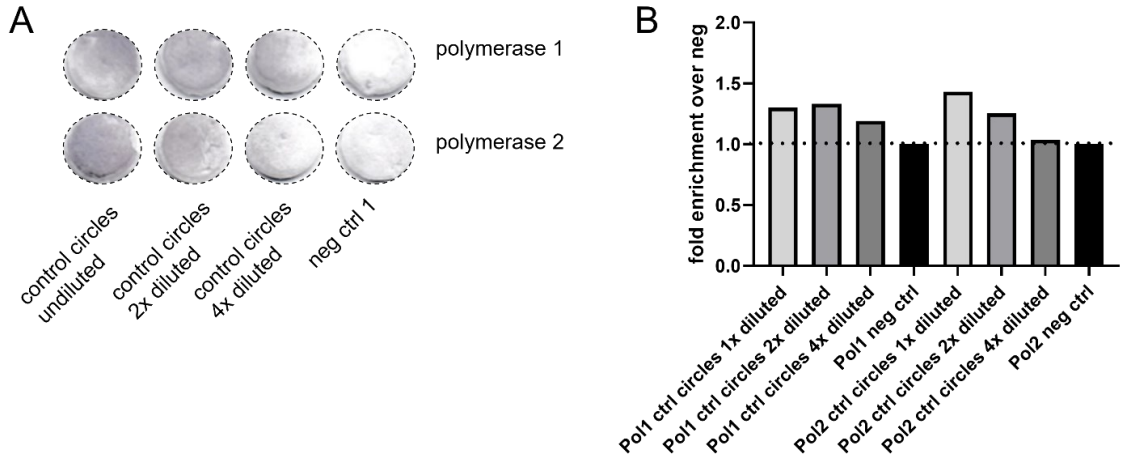


Figure 26 Polymerases shipped at different temperatures were functional.
A) Filter staining of droplet microfluidic assay of (left to right) control circles undiluted, 2x diluted, 4x diluted and negative control (neg ctrl) 1. For the assays in the upper row polymerase 1, stored at -20°C, and for the lower row polymerase 2, stored at 5°C was used.
B) Fold enrichment over the staining of the individual neg ctrl, measured as brightness, dotted line indicates value of neg ctrl set to 1.
N=1

4.4 Comparison of different sample dilutions

We examined the influence of sample dilution on the color intensities of filters and compared a diminished sample dilution against the original dilution. For the original concentration homogenized saliva samples were diluted 50fold in PBS (10µl saliva in 490µl PBS), whereas with the intention to improve the signal strength on the filters a ten times lower saliva dilution was analysed. To ensure that the reduced dilution would not influence the integrity of the negative control regarding the background noise, two negative controls were compared with the same dilution factors as the samples. The experimental setup was first and second negative control, four samples of four individuals (sample 5-8), of which one (sample 8) was processed once with the initial dilution and again with a ten times lower dilution, an additional negative control processed with ten times lower dilution and positive control 3 (Fig. 27A). As seen in Figure 23 B, the lower dilution had no effect on the enrichment over the original negative control neither of the positive sample nor of the lower diluted negative control compared to initial saliva dilutions. For further experiments we therefore chose a 50-fold dilution of saliva samples. In addition, the overall weak signal intensity on filters except those from positive controls is most obvious in Figure 23B, depicting barely any enrichment in none of the samples. The presented assay demonstrated no correlation between filter intensities of saliva samples and the associated parasitaemia observed in blood smears ($R^2=0.006391$, p-value of 0.8983, Fig. 27C). This phenomenon held validity across various degrees of parasitaemia ranging from 437p/µl (sample 8), 962 p/µl (sample 6), 2739 p/µl (sample 7), 6869 p/µl (sample 5) as illustrated most apparent on Fig. 27D.

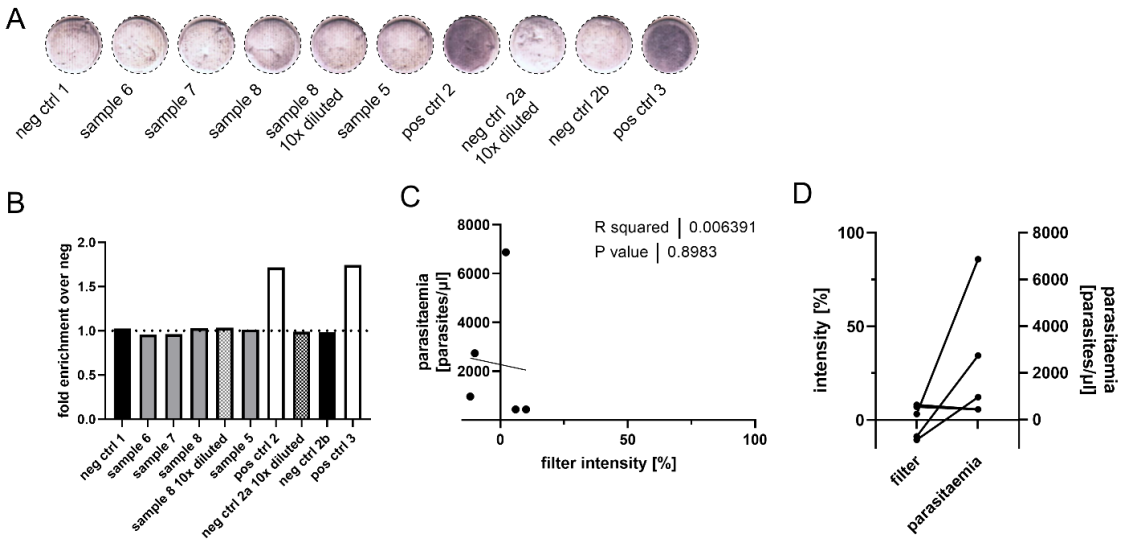


Figure 27 No difference in staining intensity was observed when samples were ten times diluted.
A) Filter staining of droplet microfluidic assay of (left to right) negative control (neg ctrl) 1, sample 6-8 in initial dilution (1:5), sample 8 10x diluted (1:50), sample 5 (1:5), positive control (pos ctrl) 2, neg ctrl 2a 10x diluted (1:50), neg ctrl 2b (1:5), pos ctrl 3.
B) Fold enrichment over the staining of the averaged neg ctrl for samples of initial concentration (1:5), as well as over 10x diluted neg ctrl for sample 8 10x diluted.
C-D) Filter intensity in percentage of positivity (normalized to neg and pos ctrl) is correlated to the microscopically counted parasitemia by standard thick blood smear procedure (number of parasites per µl of blood [parasites/ µl]).
C) Linear regression analysis with an R^2=0.006391 and p-value of 0.8983.
D) Correlation of individual patient values of filter intensity and microscopically counted parasites.
N=1, 4 individual samples

4.5 Alternation of HRP-concentration

Different HRP-concentrations were tested and compared on a positive sample (sample 14 boosted) and a negative control. With 8µl HRP per 1ml blocking buffer being the initial concentration, also concentrations of 2µl/ml, 4µl/ml, 16µl/ml and 32µl/ml were assessed (Fig. 28A-C). When normalized over the corresponding negative control filter, the positive sample with 2µl/ml, 4µl/ml and 32µl/ml HRP showed notable enrichment of intensity (Fig. 28B). It could also be seen that with increasing HRP concentrations, the background noise on negative control increased. Measured as filter brightness, this affected most strongly negative controls with 16µl/ml and 32µl/ml HRP administered (Fig. 28C). The brightness-

to-noise ratio was most favourable when 2µl/ml to 8µl/ml HRP were used. Lower HRP concentrations were thereupon retested in the subsequent assay.

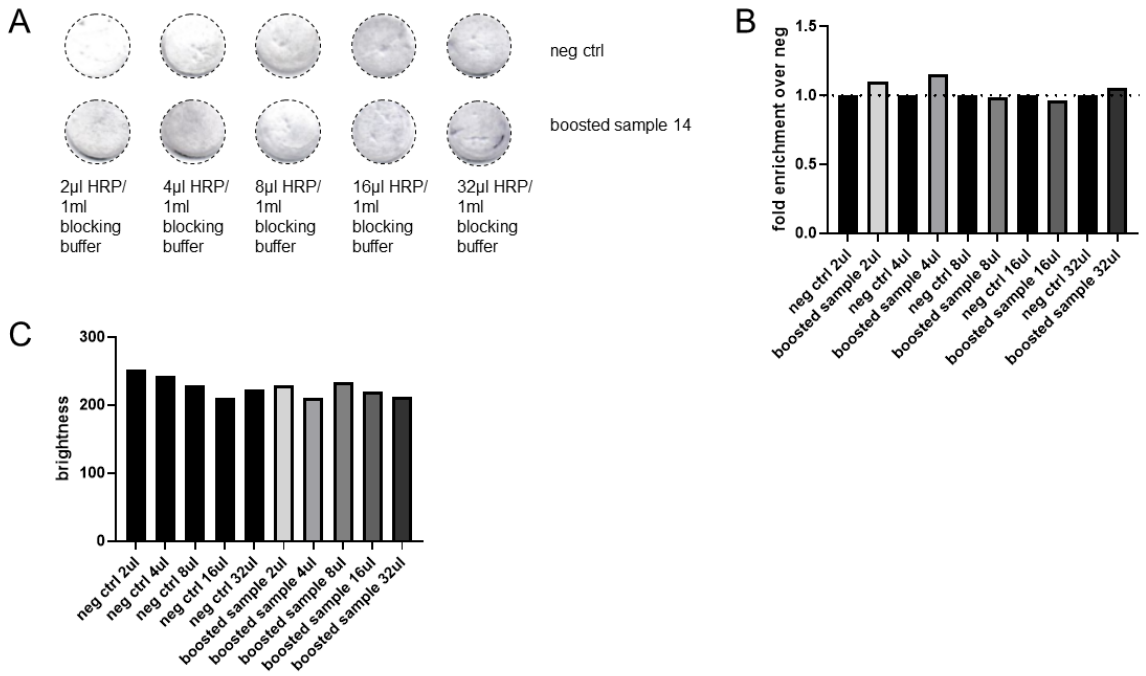


Figure 28 Increased HRP concentration enhanced staining intensity, but also enhanced background staining intensity.
A) Filter staining of droplet microfluidic assay of upper row the negative control (neg ctrl) and bottom row boosted sample 14 with from left to right increasing concentrations of HRP from 2µl to 32µl.
B) Filter staining measured as brightness is plotted as fold enrichment over the corresponding neg ctrl.
C) Measurement of filter brightness.
N=1, 10 individual samples

Thereafter, the assay was conducted in three independent repetitions comparing again 2µl, 4µl and the initial concentration of 8µl HRP per milliliter blocking buffer to verify the effect of lower HRP concentrations. The experimental setup contained a negative control and boosted sample 14 (Fig. 29A-C). A significant fold enrichment over negative controls was observed for all three different administered HRP concentrations (Fig. 29B). But when measured as brightness, the ratio between negative controls and positive samples was low with the use of 2µl/ml HRP (Fig. 29C) and positive and negative samples could barely be

distinguished on filters (Fig. 29A). We assumed that 2µl HRP per mililiter blocking buffer was insufficient for adequate colour reaction on filer membranes and following designed more assays to investigate HRP concentrations of 4µl/ml and 8µl/ml.

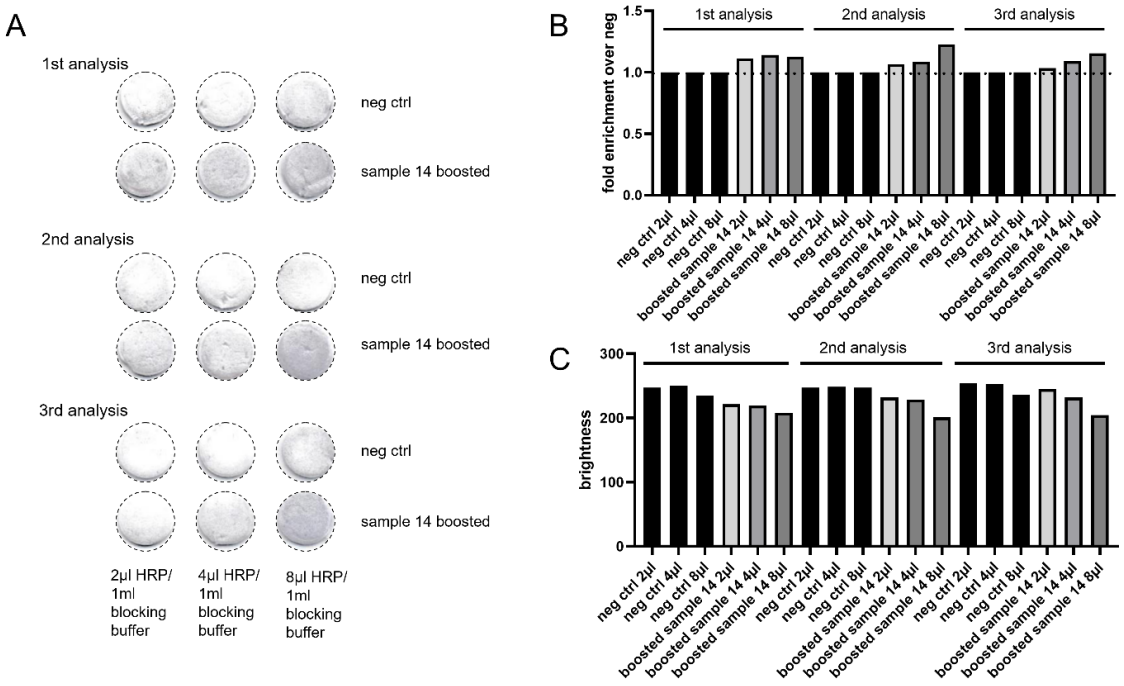


Figure 29 Higher concentrations of HRP allowed to distinguish the staining of positive samples from background.
A) Three repetitions of droplet microfluidic filter stainings of upper row the negative control (neg ctrl) and bottom row boosted sample 14 with from left to right increasing concentrations of HRP from 2µl, 4µl to 8µl.
B) Fold enrichment over the staining of the corresponding neg ctrl, measured as brightness, dotted line indicates value of neg ctrl set to 1.
C) Measurement of filter brightness.
N=3

Subsequently, the assay was performed with four fresh saliva samples and in two readouts, one with 4µl HRP per 1ml blocking buffer (upper row) and one with 8µl HRP/ml blocking buffer (lower row) (Fig. 30A). All required controls were included. As shown in Figure 30B, fold enrichment over negative controls was much higher when 8µl HRP/ml blocking buffer was used (right side). With the lower HRP concentration, almost no signal enrichment was reached in this assay except for positive controls. The obtained filters of negative controls and all

samples in the experiment with 4µl HRP resulted in similar staining and could not be differentiated (Fig. 30A). Both analyses did not show any correlation between filter intensity of saliva samples and correlating blood count parasitaemia (Fig. 30C-E). While the use of 8µl HRP yields improved results, there was still no significance (R^2 = 0.1072, p value 0.6725, Fig. 30D). Figure 30E shows that for all levels of parasitaemia ranging from a negative sample (sample 22) to 6755p/µl (sample 20) in both assays (dark and light colours), no agreement was seen. Interestingly though was that sample 20 which had the highest blood count parasitaemia of the here analysed samples, reached the highest filter intensity in the second analysis with 8µl HRP. According to our results we identified 8µl HRP per mililiter blocking buffer the most favourable concentration regarding signal strength and background noise.

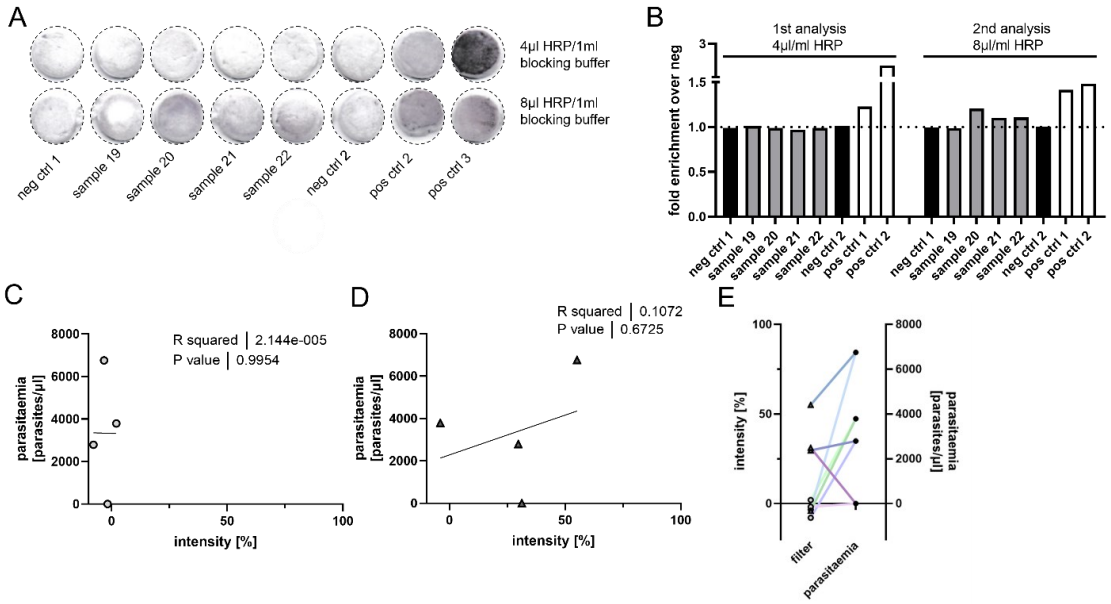


Figure 30 The usage of 8µ/ml HRP improved the resolution of the droplet microfluidic assay.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample number 19 to 22, neg ctrl 2, positive control 2 (pos ctrl 2), pos ctrl 3, upper row with 4µ/ml HRP and lower row with 8µ/ml HRP.
B) Filter staining measured as brightness is plotted as fold enrichment over the average of neg ctrls.
C-E) Filter intensity in percentage of positivity (normalized to neg and pos ctrl) is correlated to the microscopically measured parasitemia by standard thick blood smear procedure (number of parasites per µl of blood [parasites/ µl]).
C-D) Linear regression analysis for C) 1st analysis, depicted in circles and D) 2nd analysis, depicted in triangles.
E) Correlation of individual patient values of filter intensity and microscopically measured parasites. 1st analysis indicated by lighter colors, 2nd analysis by brighter colors of sample 19 in green, sample 20 in blue, sample 21 in purple and sample 22 in pink.
N=1, 4 individual samples

5. Testing the samples stability

To find out how long samples can be stored in the fridge before being analysed, the following experiment was designed. Samples were collected and tested after one and again after three days. Meanwhile, samples were stored in the fridge at a temperature of 4°C. Ten samples were analysed together with negative control 1 and 2, positive control 2 and in the first assay additionally with positive control 3 (Fig. 31A-C). Filters of samples resulted in different colour intensities, negative

controls were as clear as we were generally able to achieve and the positive controls were intensely coloured (Fig. 31A). Accepting the limitations that were experienced during all the assays, it can be stated that the assay on day one was successfully conducted and corresponded our expectations. One day after collection, a positive enrichment over negative controls could be seen for all samples except sample 9 (Fig. 31B_1), whereas on day three after collection enrichment over negative controls was generally weaker for all samples and no enrichment was measured for five samples (sample 10,12-15) (Fig. 31B_2). Filter brightness increased after storage of samples when comparing d1 to d3, meaning colour intensity was reduced and brightness of filters belonging to samples was less variable than when analysed one day after collection (Fig. 31C). We refrained from re-testing the samples at a later point of time since signals decreased notably in a two-day period and assumed that for improved assay quality samples should be processed immediately after collection.

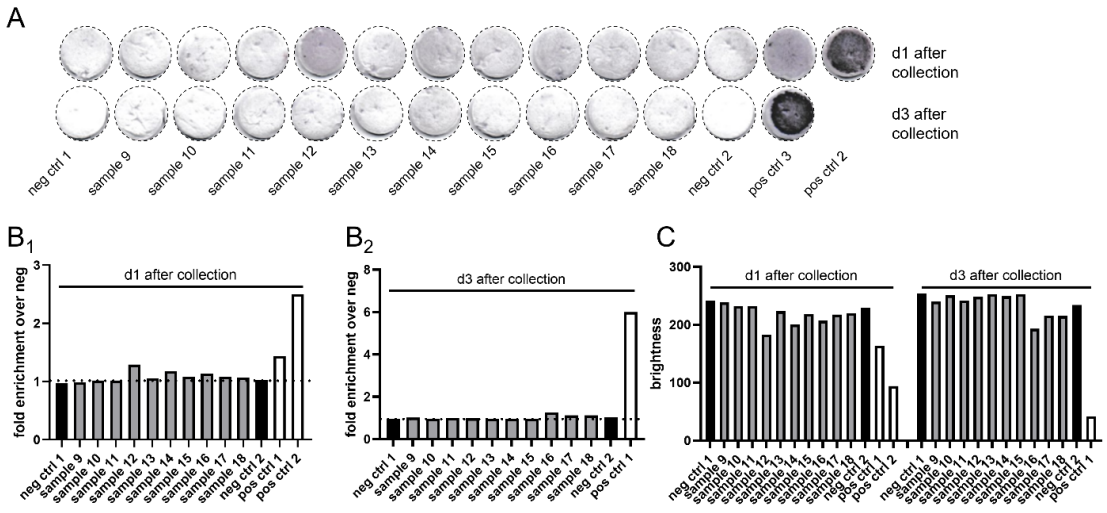


Figure 31 Intensity of filter staining decreased with prolonged storage.
A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample number 9 to 18, neg ctrl 2, positive control 3 (pos ctrl 3), pos ctrl 2, upper row samples processed 1 day (d1) after collection and lower row samples processed 3 days (d3) after collection.
$B_{1, 2}$) Filter staining measured as brightness is plotted as fold enrichment over the average of neg ctrls for samples processed B_1) d1, or B_2) d3.
C) Measurement of filter brightness.
N=1, 10 individual samples

6. Summary of results

Using the final protocol as elaborated during the process of optimisation, two independent final experiments on two different days with ten different samples were conducted (Fig. 32A-D). Figure 32A shows the experimental setup for both assays with negative controls, ten individual samples each and positive controls.

Signal enrichment of samples could be obtained in both set ups (Fig. 32B), however especially in the first assay including sample 23-32 signal intensity of samples was rather weak when compared to negative controls, once more outlining a general limitation we encountered throughout the process of optimisation. One improvement we were able to achieve is the lower background staining of negative controls with negative controls processed first and last resulting in almost the same brightness in both assays (Fig. 32C). Furthermore, cumulative contamination seen as increasing signal intensity according to the processing sequence of samples was no longer evident (Fig. 32 A-C). Despite optimised colour intensity and reduced background noise achieved with the final protocol, no correlation between signal intensities of filters belonging to saliva samples and matching microscopically estimated blood smear parasite counts could be observed (Fig. 32D, E). Our data did not show statistical significance (R^2=0.002459, p value 0.8355, Fig. 32D) in correlation of filter staining and blood count parasitaemia and as presented in Figure 32E, better agreement could be seen for low parasitaemia. However overall the droplet microfluidic assay did not correlate with blood smear microscopy.

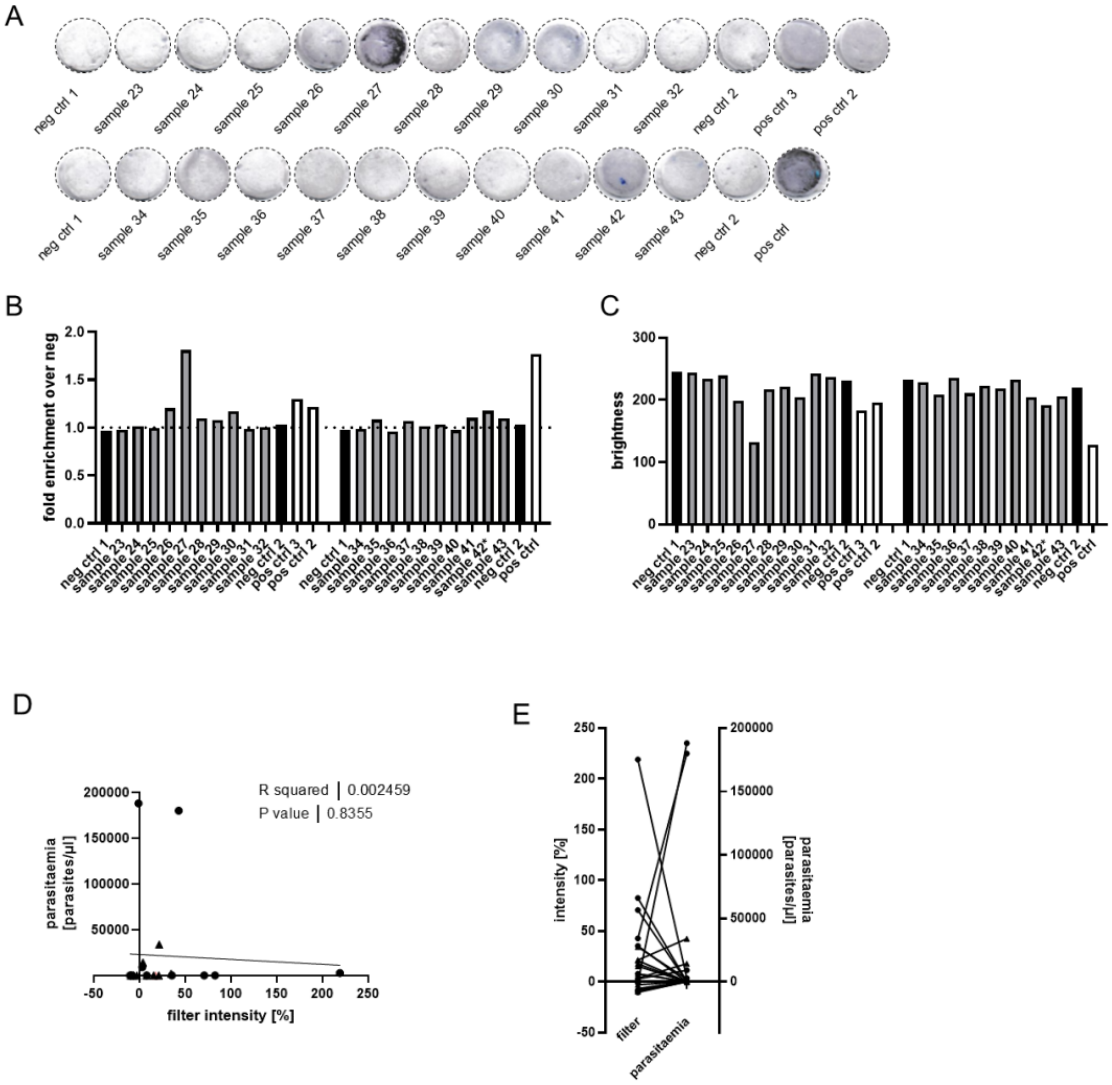


Figure 32 Despite the technical optimization of reduced background and enhanced sample staining intensity, the results of the microfluidic droplet assay did not correlate with the degree of parasitemia in the blood.

A) Filter staining of droplet microfluidic assay of (left to right) negative control 1 (neg ctrl 1), sample number 23-32 (upper row), sample number 34-43 (lower row), neg ctrl 2, positive control 3 (pos ctrl 3), pos ctrl 2.

B) Filter staining measured as brightness is plotted as fold enrichment over the average of neg ctrls.

C) Measurement of filter brightness.

D-E) Filter intensity in percentage of positivity (normalized to neg and pos ctrl) is correlated to the microscopically measured parasitemia by standard thick blood smear procedure (number of parasites per µl of blood [parasites/ µl]).

D) Linear regression analysis with an R^2=0.0025 and p-value of 0.8355.

E) Correlation of individual patient values of filter intensity and microscopically measured parasites.

N=1, 20 individual samples

IV Discussion

Malaria, a parasitic disease with a potentially deadly outcome if left undiagnosed and untreated, still constitutes a serious health risk for half of the world's population. Despite the progress that was achieved during the last decades concerning disease control, diagnostics and treatment, still more than 200 million malaria cases are reported worldwide annually (WHO 2023d).

Moreover, the COVID-19 pandemic has had both direct and indirect effects on malaria elimination efforts (Zawawi, Alghanmi et al. 2020). One notable consequence has been the disruption of malaria elimination efforts, as resources and attention have been diverted to combat the novel coronavirus. With health systems overwhelmed by the influx of COVID-19 cases, many countries had to redirect personnel, finances, and medical supplies to cope with the demands of the pandemic and strained the capacity of malaria control programs. Estimates for the early phase of the pandemic predicted an increase in malaria case incidence by 21.5% and a doubling of malaria related deaths compared to baseline levels in malaria endemic countries due to interrupted ITN and antimalarial drug distribution in Africa (Weiss, Bertozzi-Villa et al. 2021). Although this worst-case scenario did not manifest, global malaria deaths increased by 12% in 2020 compared to 2019 and 68% (69,000 deaths) of these were related to disrupted services due to COVID-19 (WHO 2021d). Furthermore, the pandemic disrupted routine healthcare services, including malaria diagnosis and treatment. Lockdowns, travel restrictions, and fear of infection led to a decline in healthcare-seeking behaviour for febrile illnesses, including malaria. The closure of health facilities and restrictions on movement hindered individuals' access to malaria testing and treatment services (Heuschen, Abdul-Mumin et al. 2023,) (Ilesanmi, Afolabi et al. 2021). Additionally, the economic repercussions of the pandemic have had indirect effects on malaria elimination. The economic downturn triggered by COVID-19 has strained the financial resources available for public health initiatives to control malaria. (Anyanwu and Salami 2021).

Malaria poses a considerable challenge even in non-endemic regions. The increasing global movement of individuals has led to a notable surge in reported cases of imported malaria over the past few decades. Moreover, climate change increases the geographical extent of malaria transmission in some regions and may lead to an increase in cases of airport malaria (Alenou and Etang 2021).
In spite of the concerted achievements realized over preceding decades in the domain of malaria control, sustained and ambitious endeavours remain imperative for the attainment of efficacious and enduring malaria elimination. The advent of unanticipated events, exemplified by the COVID-19 pandemic, has the potential to undermine years of progress. Persistent vigilance is requisite in the face of continuously emerging challenges, demanding innovative approaches for their management. Malaria persists as a formidable global health challenge, necessitating an ongoing commitment to address its multifaceted complexities.

1. Evaluation of the droplet microfluidic assay

Improved diagnostics, which are affordable, easy implementable and characterized by high diagnostic performance are important for continued progress in the control of malaria (Oyegoke, Maharaj et al. 2022). One among the newly developed diagnostic methods, the droplet microfluidic assay for malaria diagnostics in human saliva, is being presented in this work. While conventional malaria diagnostic tests require a blood specimen, the here presented method is based on diagnostics from saliva samples, which has the potential to importantly simplify malaria diagnostics due to the many advantages that saliva possesses as biological specimen and the non-invasiveness of obtaining saliva.
The test principle was developed by an academic collaboration in Denmark and yielded promising results in its diagnostic performance (Juul, Nielsen et al. 2012), (Hede, Fjelstrup et al. 2018). This work describes the next step in the development of this test, which constitutes the transfer of the test to an endemic

setting to evaluate its implementability and diagnostic performance. In addition, this work systematically evaluated the pitfalls of the test and aimed to improve its diagnostic workflow.

With the first assays performed in Gabon (Figure 15), we observed two major technical limitations. Firstly, negative controls showed a high background. Secondly, the signals of saliva samples that were malaria positive according to corresponding thick smear blood microscopy showed relatively weak colour intensities. The combination of these two unexpected technical obstacles made it often impossible to distinguish between positive and negative samples. From the results we obtained from the first assays we concluded that the technical development of the microfluidic assay was obviously still insufficient for the tropical setting precluding to the start of a larger field testing of this diagnostic assay. We therefore concentrated on the assay optimisation of the test to facilitate the further development of the assay. In the following chapter we interpret our findings and advancements and reflect on the evaluated test in the context of recent malaria research and required developments for malaria elimination.

1.1 Reducing background activity

We observed an increasing colour intensity of filters according to the order of processing and suspected an accumulative contamination caused by parasite components. Gradually increasing washing steps of materials were applied and our investigations indicated that among the various applied washing methods NaOH demonstrated superior efficacy (see Figure 17-20). Sodium hydroxide effectively eliminated nucleic acid constituents from plastic ware by denaturation of double-stranded DNA or RNA and the hydrolysis of phosphodiester bonds within the nucleic acid backbone (National Center for Biotechnology Information 2023). The adoption of this mechanism has garnered increased attention in recent times. In response to the scarcity of laboratory resources in numerous industrialized nations, particularly evident during the initial phase of the COVID-

19 pandemic, laboratories were compelled to devise strategies for the extended utilization of specific supplies, such as PCR filter tips. Kumar et al. (2023) elucidated in their study a procedural framework involving a sequence of washing steps with distilled water, NaOH, and either hydrogen peroxide or ethanol, enabling the reutilization of disposable plastic ware (Kumar, Shamni et al.).

We also examined the effect of the use of single use consumable on the technical performance of the assay. Our findings indicated that employing single-use syringes and single-use microfluidic chips led to improvements in the background intensity of negative controls (Figure 21-23). Notably, the substitution of inlet tubings, as opposed to their washing with sodium hydroxide, did not improve readouts. The use of disposable materials for all steps was however judged as not feasible due to the limited resources in the setting. Furthermore, a predilection for disposable materials characterized by high turnover was deemed unfavourable for the future implementation of the diagnostic methodology within the constraints of a resource-scarce context.
Although we were not successful in achieving optimal clear negative controls a substantial overall improvement was observed after modification of the processes. Cross contamination between samples was minimized and the two negative controls in one setup resulted in almost the same colour intensity. Nevertheless, taken all together, we cannot sufficiently explain why this problem of background colouration of negative controls occurred in the tropical setting while never observed in the assays when performed in Denmark.

1.2 Ensuring reagent integrity

We observed that several saliva samples with microscopically confirmed malaria infections in matching blood samples resulted in relatively weak colour reactions in the visualization process and its following colorimetric readout. Differentiation between positive and negative saliva samples was thereby limited. This was again a surprising finding, since this obstacle had never occurred in Denmark before.

Our first hypothesis was that one or more reagents had somehow degraded in their quality in the process of transportation between Denmark and Gabon since all reagents involved in the performance of the experiment were shipped from Denmark.

We were able to proof that the TMB used in Gabon showed to be fully functional in Denmark and storage conditions of -80°C should not have any impact on the nucleotides' integrity. As presented on Figure 26 we confirmed the functionality of both stocks of phi-polymerases and concluded that storage temperatures between -20°C and 5°C were appropriate for the phi-29 polymerase selected for the microfluidic assay. The experiment depicted on Figure 26 also showed that the polymerase was still highly active providing a sufficiently coloured DNA filter. Therefore, given that the amount of circular DNA produced by the target enzyme was sufficient, the following steps of the assay should have proven unproblematic.

1.3 Adjustment of reagent concentrations

By systematic evaluation of repetitive experimental settings, we were able to define optimised reagent concentrations. Our investigation revealed that exceeding substrate concentrations of 1 µl per 400 µl lysis buffer resulted in elevated background levels in negative controls (refer to Figure 25).

In addition, further experiments conclusively demonstrated that the analysis of higher sample volumes, being blood or saliva, may lead to higher diagnostic sensitivities (Dowling and Shute 1966), (Ghayour Najafabadi, Oormazdi et al. 2014), (Imwong, Hanchana et al. 2014), (Hofmann, Mwingira et al. 2015). We tested the effect of a tenfold lower saliva dilution (see Figure 27), which exhibited no discernible impact on either signal intensity or negative controls, therefore, the initial 50-fold dilution of the saliva samples was adopted in the final protocol. While the possibility of testing even lower sample dilutions was considered, it was ultimately dismissed as a certain liquescency was considered necessary for the

appropriate processing of saliva samples, particularly as they are propelled through the chip.

Our findings also supported the adoption of undiluted control circles to yield optimal results in terms of signal intensity (refer to Figure 26). Additionally, an exploration of both higher and lower concentrations of HRP was undertaken to ascertain a concentration aligning with our criteria for high signal intensity in positive samples and minimal background in negative samples and controls (see Figure 28, 29, 30). Among the tested concentrations, an optimal concentration of 8 µl HRP per milliliter of blocking buffer was identified.

In the course of varying reagent concentrations, we demonstrated that the overall signal strength achieved in Gabon was comparable to that observed in Denmark. Consequently, we concluded that the overall reaction process was comparable in the new experimental setting.

1.4 Testing sample stability

We showed that already after three days of sample storage overall signal intensity declines substantially (Figure 31). These findings suggest that samples collected in Gabon are stable for around one day under cooled conditions and should be analysed as soon as possible after collection. In similar previous experiments carried out in Denmark it was demonstrated that cooled but unfrozen samples result in stronger signals than frozen samples. Furthermore, the samples analysed in Denmark were shown to decline in signal intensity over time, but an overall strong signal could be observed for at least 45 days when stored in a fridge, as presented on the chart in figure 33 (Hede, Fjelstrup et al. 2018).

We assume that the sample stability was influenced by the overall weak signal strength of the assay that was observed in the test setting in Gabon. As the signals obtained immediately after collection were in general of lower intensity, the decline of signal strength was more considerable in Gabon than in Denmark.

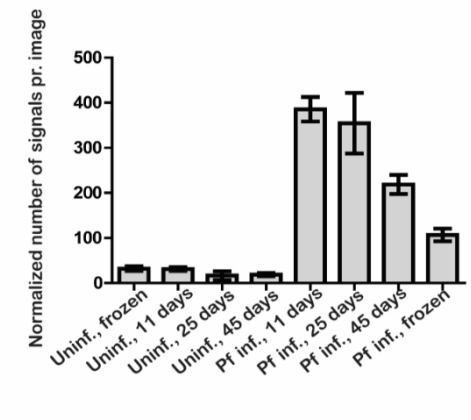


Figure 33 Samples tested in Denmark are more stable over time
Bar chart showing the results of analysing a saliva sample that had either been frozen upon arrival or kept at 4°C for 11, 25 or 45 days. The error bars on the bars representing the results of testing the positive samples therefore show the standard error of measurement for 16 randomly selected microscopic images.

Source: Hede, Fjelstrup et al. 2018

1.5 Comparison of results obtained in Denmark and Lambaréné

Reviewing the results, which we have obtained in Lambaréné after the technology transfer and during this project, we conclude that the technology transfer did not succeed as anticipated. While the test set-up worked consistently in Denmark, even after several optimisation steps the assay was not performing sufficiently well in Gabon. The overall good test performance observed in Denmark with shipped saliva samples was therefore not reproduced in the setting of Gabon.

Comparing the first and the last assay performed in Lambaréné (Figure 15 and Figure 32), the progress, which was accomplished by this project, can be directly observed. We minimized background colouration as represented by the similar colour intensity of first and second negative controls. Furthermore, an overall lower background intensity on negative samples was achieved. Nevertheless, with regards to the two final experiments that were conducted according to the finalized protocol as it was elaborated during the course of this project, the colour intensities on the filters were not predictive for the microscopically determined malaria positivity and negativity of the matching saliva samples.

Analysing the outcomes described in the two different countries it became evident that there were several differences regarding the results of the assays, which we cannot fully explain. While we were able to rule out several hypotheses of why the assays did not perform well, the final reason became not identifiable. We therefore conclude that the microfluidic assay is not yet sufficiently technically optimised for the new setting. At this point, an evaluation of the performance of the new method or further comparative testing was therefore not yet possible.

During the process of technology transfer of the droplet microfluidic assay and its advancing implementation in the malaria endemic region of Gabon, many lessens have been learned. Controlled shipment of materials to ensure reagent functionality is essential for stable test performance at the site of implementation. The use of disposable consumables has to be considered carefully with regards to long-term supply of items at the testing facility, especially when shipments are logistically challenging and time consuming. The availability of adequate storage facilities under extreme climatic conditions on the other hand can be scarce. Both equipment and samples can be sensitive to heat and humidity leading to impaired results, therefore robust assays unsensitive to climatic influences are necessary. According to the findings derived from the conducted experimental analyses, the effect of altered saliva dilutions, substrate concentrations and applied quantities of TMB on staining intensity of filters were assessed at the site of intended implementation. Processing unfrozen saliva samples promptly after collection and repeatedly over a specified duration, our study explored sample stability and identified a reduced period of storage viability in the new context.
Based on the results we obtained from our study, the testing principle was further refined, and a new optimised prototype of the assay was developed in Denmark. Currently, this prototype is undergoing testing with samples from Gabon in Denmark, demonstrating further improved characteristics. The subsequent field evaluation is scheduled for the upcoming years and will once again involve a pilot implementation of the test in Gabon before it is tested in a prospective study.

2. The need for improved diagnostic methods

2.1 The value of diagnostic testing

Accurate diagnostic testing is a necessary requirement for adequate patient management to confirm or rule out a clinical diagnosis, as well as to conduct screening programs to discover asymptomatic cases to reduce disease transmission. Furthermore, reliable diagnostics are important for surveillance programs, as constant monitoring allows to capture the success and progress of interventions (Peeling, Smith et al. 2006). In summary, the point-of-care test may be particularly suitable for the diagnosis of symptomatic patients and on the other hand to detect asymptomatic, sub-microscopical infections to control and ultimately assist in eliminating malaria. (Zimmerman and Howes 2015).

It was repeatedly shown that particularly in high transmission regions, malaria is widely over-diagnosed and patients presenting with fever or history of fever are very likely to be treated with antimalarial medication, irrespective of laboratory confirmation (A-Elgayoum, El-Feki Ael et al. 2009), (Leslie, Mikhail et al. 2012). Over-diagnosing malaria leads however to various consequences for malaria control programs as well as for public health system. Firstly, the economic consequences for treating patients not actually suffering from malaria infections with antimalarial drugs are significant as money is spent on medications which are not needed for the actual cause of disease, burdening patient's economic situation as well as the public health sector (Njuguna, Menge et al. 2015). Secondly, the widespread and often incorrect prescription of antimalarial medications poses an unnecessary further risk for the emergence and selection of drug resistant strains of *Plasmodium* species. Particularly in Asia, more and more countries are facing the difficulties of multi-drug resistance, which puts lives in danger as antimalarial drugs are becoming less or even not effective and untreated malaria infections can result in death (Fairhurst and Dondorp 2016). Finally, over-diagnosing malaria involves the risk of failing to recognize a different underlying cause of fever, which might be curable itself but might result in a life-

threating situation if not adequately treated (Manguin, Foumane et al. 2017), (Orish, Ansong et al. 2016).

Even though over the past years more and more advanced methods for the diagnosis of malaria have been developed, high quality, specific and sensitive malaria diagnostics remain nevertheless a challenge.(Kasetsirikul, Buranapong et al. 2016). As mentioned above, adequate diagnosis is essential to successful disease control and as outlined in the latest WHO guidelines published in 2023 all suspected malaria cases should receive a parasitological test with either blood smear microscopy or a rapid diagnostic test to confirm the diagnosis before initiating treatment (WHO 2023c).

2.2 Discussion of available diagnostic methods

Despite important advances in the detection of malaria (Hede, Fjelstrup et al. 2018), WHO still defines blood smear microscopy as the gold standard of clinical malaria diagnostics (WHO 2023c). Advantages of light microscopy for malaria diagnosis include moderate costs, high sensitivity, detection of all *Plasmodium* species and the possibility of parasite density quantification and thereby monitoring of treatment efficacy (Oyegoke, Maharaj et al. 2022). However, malaria microscopy also has important limitations. Even for an expert microscopist, the parasite detection limit is ~10 parasites/µl blood, under typical field conditions the sensitivity is lower with a threshold of approximately 100 parasites/µl peripheral blood. These relatively high detection limits compared to other methods might lead to false negative test results in case of low parasitaemia. The minimum parasitaemia observed microscopically in our specimens was 47 parasites/µl, a value consistent with the anticipated standards set forth by the WHO for proficient microscopists. As the readout depends on the proficiency level of performing staff, intense training is required for high quality results. Another limitation to accurate microscopic detection may the presence of mixed species infections which are frequently misdiagnosed due to the morphological similarities among early parasite stages (Fitri, Widaningrum et al. 2022). Among the 43 samples we collected, one mixed infection with *Plasmodium*

falciparum and *Plasmodium ovale* was detected. No further diagnostic assay was performed to differentiate *Plasmodium* species, therefore the occurrence of other mixed infections cannot be reliably excluded. Moreover, the quality of the staining critically influences the microscopic readout. Giemsa stain on blood smears requires careful handling to mitigate the risk for artefacts that may be misinterpreted as parasites. From a technical standpoint, Giemsa staining should be performed in a basic safety cabinet, which is not readily available everywhere, as is reliable electric power supply for the microscopes (Ohrt, Purnomo et al. 2002).

In order to reduce human error and circumvent the need for extensive training new diagnostic approaches make use of smartphone-based applications. Yu et al. (2023) compared the so called ‘Malaria Screener’ app for thick smear microscopy with results obtained by trained microscopists (Yu, Mohammed et al. 2023). A semi-automated method is used where a smartphone is connected to a microscope and fields of vision of the thick smear are presented to the application, which can detect malaria parasites using machine learning and computer vision algorithms. With an accuracy of 74.1%, sensitivity of 100% and specificity of 51.1% the results equal the WHO level 3 competence requirement. During post-study modifications a WHO level 1 competence was reached (Yu, Mohammed et al. 2023). As machine learning is constantly improving, so will these apps, indicating that in future a microscope device linked with a phone might become a reliable diagnostic tool that does not require extensive training of microscopists anymore.

According to WHO the presence of malaria parasites shall be conformed prior to treatment initiation using a parasitological test (WHO 2023c). RDTs require only limited training of personnel, no specialized test environment, provide an immediate result within a few minutes and are cost-effective. However, there are certain limitations that warrant consideration. The majority of RDTs are based on the presence of histidine-rich-protein-2 (HRP-2) antigen in blood of infected individuals (Rachid Viana, Akinyi Okoth et al. 2017). Other commonly used antigens include the plasmodial lactate-dehydrogenase (pLDH) or the plasmodial

aldolase (Mathison and Pritt 2017). In contrary to the pLDH and the plasmodial aldolase, the HRP-2 gene is specific to *Plasmodium falciparum* species, which in consequence means, that other species are not detected.

HRP-2 has a long clearance period, during which time the antigen remains in the human bloodstream, resulting in a high rate of false-positive RDTs in patients having recently received antimalarial treatment especially in high-transmission areas (Mouatcho and Goldring 2013). The median time for a positive WHO certified HRP-2 based RDT was 35 - > 42 days as found by Mouatcho et al. (2013). This high false-positive test rate does not only lower specificity of RDTs, but also hinders the diagnose of a reinfection within the clearance time. In the course of our investigation (n= 43 samples) 7 RDTs (16.3%) exhibited false positive outcomes in comparison to results obtained by blood smear microscopy. This discrepancy may be attributable to the persistence of HRP-2 in the patients' blood subsequent to the clearance of parasites from blood and roughly consistent with a positive predictive value of HRP-2 based RDTs of 69% in Gabon (Mischlinger, Dudek et al. 2021).

Another layer of complexity is added by the increasingly frequent deletion of HRP-2 and HRP-3 encoding genes in the parasite’s genome. Without the *PFHRP2* gene, malaria parasites do not express the histidine-rich-protein, which is otherwise found in all different blood stages of *Plasmodium falciparum*. The *PFHRP3* gene is a structural paralog to *PFHRP2* (Rachid Viana, Akinyi Okoth et al. 2017). Consequently, RDTs targeting HRP-2 might give false negative results due to the presence of malaria parasites lacking HRP-2 and/or HRP3.

So far, the majority of studies showing the extent of the pfHRP-2/3 gene deletion have been conducted on the American continent in countries such as Colombia, Brazil or Peru but further studies are increasingly conducted in sub-Sahara Africa. In the Peruvian Amazon 41% (Dorado, Okoth et al. 2016), in Ghana 18-50% (Amoah, Abankwa et al. 2016) and in Mali 50% (Koita, Doumbo et al. 2012) of samples tested exhibited the deletion of PFHRP-2/3 related genes in *Plasmodium falciparum*. Among the 43 here performed HRP-2 RDTs, we did not find false negative RDTs when compared to microscopy. These findings align

with the results of various studies on *PFHRP2* gene deletions in Gabon estimating the frequency of a *PFHRP2* gene deletion to approximately 1% in the Gabonese population (Krueger, Ikegbunam et al. 2023),(Kreidenweiss, Trauner et al. 2019). RDTs detecting pLDH show in general a lower specificity and sensitivity than those based on HRP-2, but unlike the latter, the pLDH elimination time is shorter and most rapid tests remain positive only for a median of 2 days (Grandesso, Nabasumba et al. 2016).

Recently, ultrasensitive RDTs are being developed with a 10-fold lower detection limit than conventional RDTs under laboratory conditions (Owalla, Okurut et al. 2020). They are considered particularly valuable in areas approaching malaria elimination with lower parasite densities. Recent comparative studies underline their usefulness in this setting. In a study in Uganda and Myanmar a ultrasensitive RDT detected parasite densities as low as 1 parasite/µl while the conventional RDTs detection limit was 100-200/µl (with a sensitivity of 50%) (Das, Jang et al. 2017). In 2020, Owalla et al. performed a comparative analysis of a conventional, an ultrasensitive RDT, thick smear microscopy, and qPCR readouts as reference in Uganda. Particularly under field conditions, the uRDT was superior to the conventional RDT and microscopy in identifying asymptomatic malaria cases with low parasitaemias (Owalla, Okurut et al. 2020). Similarly, other studies in low-transmission settings from Cambodia (Yeung, McGregor et al. 2020) and Tanzania (Hofmann, Antunes Moniz et al. 2018) found that the novel uRDT performed slightly better than the level of cRDTs in active case detection. Regarding the 5 times higher price in relation to the incremental improvement of performance of uRDTs compared to cRDTs, ultrasensitive rapid diagnostic tests are currently unlikely to replace the conventional tests for routine clinical management at this point yet.

In the 2023 published Malaria guidelines, the World Health Organisation states that *“At present, nucleic acid-based amplification techniques have no role in the clinical management of malaria or in routine surveillance systems”* (WHO 2023c). Furthermore, immunological methods like ELISA are rather useful for epidemiological studies than for patient management. Generally spoken,

molecular methods like polymerase chain reaction (PCR) with its further developed variations like nested PCR, real-time PCR or multiplex PCR have been shown to perform excellently in terms of parasite detection limits or species differentiation (Leski, Taitt et al. 2020), (Fitri, Widaningrum et al. 2022). Conversely, they are based on advanced technology and therefore require expensive equipment, skilled technicians and time-consuming analyses. At the moment these methods are rather seen as an adjunct in malaria diagnosis than an every-day point-of-care implementation (WHO 2023c).

Other technical derivations based on molecular methods seem to have more potential in acute malaria diagnosis especially in low transmission areas. The loop-mediated isothermal amplification (LAMP) is based on isothermal DNA amplification under constant temperature. It is less time consuming than PCR technics and requires fewer materials, furthermore the readout can be performed visually with the naked eye. Different studies indicate similar sensitivities of LAMP compared to PCR demonstrating diagnostic superiority compared to conventional microscopy (Ocker, Prompunjai et al. 2016, Zhang, Chen et al. 2022) with a 5 times lower detection limit than conventional microscopy. (Antinori, Ridolfo et al. 2021). However, LAMP does not provide a quantitative test result neither a species identification. Charpentier et al. (2020) compared microscopy, RDTs and LAMP on returning travellers to confirm imported malaria (Charpentier, Benichou et al. 2020). They concluded that a combination of the LAMP method to confirm the diagnosis followed by thin blood smear microscopy for species identification and parasite density could be an ideal diagnostic pathway for non-endemic countries. LAMP requires only limited training and has the potential to detect low parasitaemia which is more prone to be overseen by less experienced microscopists. Microscopy however complements diagnostic standards in terms of parasite load and identification of the *Plasmodium* species.

The discussion of the aforementioned methodologies for malaria diagnostics reveals various inherent limitations. A number of techniques entail significant procurement and maintenance expenses, particularly molecular methods capable of detecting low parasitaemia. In regions endemic to malaria, financial

constraints often impede the adoption of costly routine diagnostic modalities (2011). The method under evaluation in this project is positioned within an intermediate range of expenditure, with higher costs than conventional rapid diagnostic tests and microscopy but below nucleic acid-based methodologies such as PCR (Hede, Fjelstrup et al. 2018). The implementation of the evaluated method necessitates several components, including syringes, microfluidic chips, diverse reagents, DNA primers, and technical instrumentation. With respect to the operational environment, the tested assay mandates a laboratory setting comprising essential equipment such as refrigerators, freezers with uninterrupted power supply, a table centrifuge, pipettes, and an incubator.

Rapid diagnostic tests currently stand out as the most versatile diagnostic medium, as they are portable and can be used directly in the field, including patients' homes, without stringent prerequisites (Moody 2002). In contrast, microscopy, widely implemented alongside rapid diagnostic tests, requires a more sophisticated laboratory setting for processes such as slide staining, drying, and readout. Nevertheless, in terms of equipment requirements, blood smear microscopy imposes a lower demand on the setup compared to the evaluated test.

An additional crucial criterion pertains to the time required for diagnosis, with rapid diagnostic tests offering timely results, slide preparation and reading necessitating approximately 30-60 minutes contingent upon the proficiency of technicians and laboratory equipment (WHO 2016b), and the evaluated test entailing an overnight duration due to incubation time. The temporal delay inherent in the diagnostic process currently constitutes a significant impediment to the microfluidic assay. Nevertheless, during the ongoing phase wherein the method is juxtaposed against the gold standard, and therapeutic decisions are predicated upon microscopy outcomes, this particular features is of little clinical importance and is seen as an intermediate step in the development process of a faster turn-around time of the improved assay.

Concomitantly, the degree of training required for the implementation of the assay represents another pivotal criterion for diagnostic test implementation. The

method in question may be regarded as intermediate between microscopy and molecular methodologies in terms of educational investment. A comprehensive understanding of laboratory standards and procedures stands as a prerequisite, thereby necessitating a more rigorous training regimen compared to the implementation of rapid diagnostic tests. Despite its inherent complexity compared to malaria slide preparation and reading, the method can be structured into discrete steps, obviating the need for sophisticated instruments or machinery.

The determination of the detection limit of parasites per microliter of blood constitutes an additional salient criterion for diagnostic tests (Mbanefo and Kumar 2020). Regrettably, the data amassed from the microfluidic assay in Gabon at present preclude any definitive assertion regarding the detection limit. Nonetheless, when conducted in Denmark, an estimated detection limit of 2 parasites/µL was observed to correspond to blood samples (Hede, Fjelstrup et al. 2018).

2.3 Biological samples for diagnostic assays

All of the above-mentioned methods have the use of blood specimens as diagnostic samples in common. Blood sampling is despite its widespread use not without inherent limitations and risks. The Institutional Review Board of the Northwestern University of Chicago states: "*The risks of taking blood include pain, a bruise at the point where the blood is taken, redness and swelling of the vein and infection, and a rare risk of fainting.*" (Northwestern University 2021). Further risks are for example a nerval lesion or potentially an accidental arterial puncture (US National Library of Medicine 2024), (WHO 2010). To carry out blood sampling always bears the risk of a needle injury, which does not only cause discomfort, but may also constitute a risk for infectious diseases being HIV or hepatitis (Himmelreich, Rabenau et al. 2013). All in all, blood testing is associated with several risks and requires an invasive sample collection method, which in consequence tends to limit patients' compliance, especially when numerous blood draws are necessary. Due to the risks and the discomfort mentioned above,

blood sampling is problematic for mass screening programs. In addition, obtaining blood samples from infants requires expertise and time and may be unsuccessful. However, both mass screening programs and targeted testing of children are playing key roles in malaria diagnostics.

Performing diagnostics based on analysing oral fluids brings therefore at least in theory several advantages: saliva collection is non-invasive, it is a medium that is easily accessible and always available, also in larger quantities and it is easy to transport and store (Chiappin, Antonelli et al. 2007), (Kaczor-Urbanowicz, Martin Carreras-Presas et al. 2017). To date, only limited information about the role of saliva as transmission route for infectious diseases is available. Some viruses such as HIV for example, which are likely to be transmitted through blood products, are proven not to be readily transmitted via saliva. Other viruses including different kinds of herpesviruses and the human papilloma virus can be found in and transmitted through human saliva (Slots 2009). Recent studies show that also potentially infective hepatitis C viruses as well as Zika viruses can be found in human saliva, but it remains questionable in what way a transmission of these viruses via saliva is relevant (Bonaldo, Ribeiro et al. 2016),(Ferreiro, Dios et al. 2005),(Suzuki, Omata et al. 2005). Moreover, since saliva sampling is a non-invasive method and does not require sharps, the risk for health care workers to acquire an infection transmitted by saliva when collecting a saliva sample is arguably lower compared to blood sampling methods. Kaczor-Urbanowicz et al. (2017) describe in their review the promising potential of saliva as a diagnostic medium (Kaczor-Urbanowicz, Martin Carreras-Presas et al. 2017). They outline three different areas, where saliva has already been shown to be a reliable tool, being dentistry (e.g. to assess the risk of caries), pharmacotherapy (e.g. to monitor drug levels) and medicine (e.g. for cancer and infectious diseases diagnostics, hormone level assessments). On the other hand, analysing oral fluids still poses various difficulties: compared to blood, saliva composition is much less stable and depends on different conditions such as the ways it is collected, age, mouth hygiene, circadian variations, etc. Up to now, standardized

sample collection methods are lacking and the known parameters are still limited (Nunes, Mussavira et al. 2015).

2.4 Saliva for malaria diagnostics

As well for the diagnostics of malaria, saliva has come into focus. Methods using saliva as diagnostic specimen can roughly be divided into nucleic acid-based approaches and antigen detection. Molecular methodologies like PCR and LAMP mainly use the small ribosomal subunit 18s rRNA as target (Murphy, Shott et al. 2013, Ghayour Najafabadi, Oormazdi et al. 2014),(Mfuh, Tassi Yunga et al. 2017)., or mitochondrial DNA (Putaporntip, Buppan et al. 2011, Lloyd, Esemu et al. 2018). which are both expressed by all Plasmodium species. Depending on reference standard methods, target and species, sensitivities for the analysis of saliva in the above mentioned studies range from 74%-95% and specificity from 94-100%. Higher parasitaemia in corresponding blood samples led to higher sensitivity (Mfuh, Tassi Yunga et al. 2017).

Common targets for antigen-based methods in saliva, typically in the form of RDTs, are HRP2 and LDH (Gbotosho, Happi et al. 2010),(Aninagyei, Abraham et al. 2020),(Apinjoh, Ntasin et al. 2021). Since no RDT for salvia diagnosis is available, all studies made use of the RDTs designed for blood specimens. Sensitivity ranges from 13.3% to 77.6%, depending on various factors such as reference method and blood contamination of samples with all over high specificity between 88.9% and 100%.

The method under investigation in this project used a different approach detecting the enzymatic activity of the plasmodial enzyme topoisomerase I (Juul, Nielsen et al. 2012). Targeting topoisomerase I has not only the advantage of it being present in all malaria species known to cause human infections but it also circumvents the risk posed by gene deletions observed for pf*HRP2/HRP3* and *pLDH*. The REEAD method, as implemented in Denmark, demonstrated a satisfying diagnostic performance by identifying 35 out of 35 malaria-positive saliva samples through a colorimetric readout (Hede, Fjelstrup et al. 2018). Intriguingly, the analysis of a malaria infected unfrozen saliva sample resulted in

higher signals even after storage of 45 days at 4°C compared to the analysis of an infected frozen sample but a decrease in signal intensity over time was observed. In a study conducted by Mfuh et al. (2017), retesting saliva samples stored at room temperature in a commercial saliva collection and DNA preservation kit after 12-13 months via nPCR revealed a decrease in DNA concentration. Nevertheless, 80% of the positive samples remained positive after a year of storage (Mfuh, Tassi Yunga et al. 2017). Their results were generally superior in terms of sensitivity achieved by nPCR compared to studies where saliva samples were stored under cooled conditions (Nwakanma, Gomez-Escobar et al. 2009) or frozen at -20°C (Pooe, Shonhai et al. 2011), aligning the outcomes of analogous investigations conducted utilizing LAMP (Ghayour Najafabadi, Oormazdi et al. 2014). A separate investigation comparing the analysis of saliva samples stored on ice with preserved saliva samples in ethanol reported superior outcomes with the inclusion of ethanol (Buppan, Putaporntip et al. 2010).

In our study conducted in Gabon, attempts to replicate similar sample stability as observed in samples stored at 4°C were unsuccessful, as samples retested after 3 days already exhibited a decline in signal intensity, rendering them unsuitable for readout. We hypothesize that the storage temperature significantly influences sample quality, thereby impacting sensitivity outcomes. The adverse impact of storing samples in frozen conditions is observed, while storage at ambient or mildly cooled temperatures is deemed advantageous. Mfuh et al. (2017) undertook their investigation in Cameroon, a country with climatic conditions similar to the direct neighbouring country of Gabon (Mfuh, Tassi Yunga et al. 2017). Consequently, the feasibility of storing saliva samples at room temperature in Gabon is plausible and warrants exploration in subsequent studies. The addition of ethanol, as suggested by Buppan et al. (2010) (Buppan, Putaporntip et al. 2010), may confer additional advantages for DNA preservation. However, the denaturing effect of ethanol on enzymes (Feng, Ma et al. 2021) precludes its adoption in our methodology.

Furthermore, saliva collection and storage modality has been proven to affect the sample quality in terms of DNA purity and concentration when extracted from saliva samples. Commercial saliva collection kits, especially with added suspension buffer, seem to provide more consistent results than native or centrifuged saliva (Garbieri, Brozoski et al. 2017). In our study, fresh saliva samples were collected in empty 50ml plastic cups with led. The evaluation and comparison of collection and storage methods was not part of our investigations and it remains unclear whether commercial kits also provide benefits for enzyme preservation and extraction. However the exploration of employing a commercial saliva collection and preservation kit for the REEAD method is proposed for future studies.

A commonly observed phenomena is that saliva based diagnostic methods achieve higher sensitivities when correlating blood samples show elevated parasite levels (Nwakanma, Gomez-Escobar et al. 2009), (Mfuh, Tassi Yunga et al. 2017), (Costa, Alvarenga et al. 2022). Concerning our data, our observations do not support a superior outcome associated with elevated parasitaemia in the assessed methodology at this stage. In order to facilitate the examination of specimens characterized by low parasitic density, a promising exploration of the susceptibility of diverse molecular targets has been undertaken (Grabias, Essuman et al. 2019), (Mercereau-Puijalon, Barale et al. 2002), (Rosas-Aguirre, Guzman-Guzman et al. 2020), (Nolasco, Montoya et al. 2021).

We followed a different approach to enable efficient detection in minimal sample volumes. Via the processing of the sample through the microfluidic chip, water-in-oil emulsions were formed and mixed with lysis buffer and the REEAD substrate forming into droplets, each functioning as a small reactor for enzyme extraction. In this context, biological reactions are accelerated and rendered more efficient, even when employing minute picoliter volumes of reagents (Juul, Nielsen et al. 2012). To allow the application of the method in low resource settings, the electric pump driven extraction step was further developed into a hand-driven extraction using a syringe-vacuum mechanism (Abate and Weitz 2011) successfully applied to the REEAD method in Denmark (Hede, Fjelstrup et

al. 2018). The formation of droplets to enhance biological reactions can also be combined with PCR in the so-called droplet digital PCR (ddPCR). In ddPCR the conventional polymerase chain reaction takes place in water-in-oil-droplets. This method has been shown to yield excellent results in terms of detection limits of malaria infections (around 11 parasites/µl) and the detection of mixed species in blood samples (Srisutham, Saralamba et al. 2017) as well as saliva samples (Costa, Alvarenga et al. 2022).

Quantitative malaria diagnostics are of importance for treatment decisions such as the estimation of severe malaria (White 2022), to monitor treatment efficacy and identify drug resistances (WHO 2016b) and reduce the overuse of antimalarial medication (Rasmussen, Alonso et al. 2022) as well as for surveillance programs and vector control to understand transmission dynamics, detect low-level parasitaemia in asymptomatic patients and identify reservoirs of infections (Sturrock, Hsiang et al. 2013). Hence, there exists a considerable scientific interest in ascertaining the quantifiability of saliva-based diagnostic methodologies and their correlation with parasitic levels in blood counts.

The majority of published studies could not find a correlation between different targets in saliva and blood, independent from the analyte, being mitochondrial Cytochrome b, 18s RNA, LDH or gametocyte-specific proteins and methodology like (nested) PCR (Mfuh, Tassi Yunga et al. 2017), (Putaporntip, Buppan et al. 2011),(Ghayour Najafabadi, Oormazdi et al. 2014), ELISA (Nambati, Kiarie et al. 2018) or antibody-based immunoassay (Tao, McGill et al. 2019). Estévez et al. (2011) compared antibody immune response to *Plasmodium falciparum* antigens via ELISA and found a correlation between serum and saliva antibody titres (Estévez, Satoguina et al. 2011). However, sensitivity and correlations fluctuated depending on the study site, type of antibody and saliva collection device and antibody titres were notably lower in saliva than in blood. Nwakanma et al. (2009) show a moderate but significant correlation of parasite DNA in saliva analysed with real-time PCR and microscopically estimated parasitaemia (Nwakanma, Gomez-Escobar et al. 2009). Various reasons are being discussed concerning the weak agreement between blood parasite levels and detection of parasite

components in saliva. One reason might be the sequestration infected red blood cells during the *Plasmodium* life cycle and therefore fluctuating parasite density in peripheral blood (Franke-Fayard, Fonager et al. 2010), (Farnert, Snounou et al. 1997).

Delley et al. (2000) examined the effect of repeated blood smear microscopy in individuals during different day times and over a period of about two weeks and found that peripheral parasite density can increase over 100-fold in the same individual over a 6-hour period. Furthermore, each study subject had at least one negative blood smear during the course of the study. Interestingly parasitaemia was significantly higher around midday, which is explained by synchronized schizont release during the intraerythrocytic life cycle (Delley, Bouvier et al. 2000). The impact of the aforementioned phenomena on body fluids other than blood, the temporal dynamics governing the detectability of parasitic components in saliva, and the time to sample negativity remain subjects of inquiry.

Our data do not demonstrate a correlation between quantified signal intensity on filters and parasite count in matching blood samples. This might be due to overall restricted test performance observed in Gabon since the REEAD method itself allows quantification of the results and has been shown to produce reliable quantitative outcome when blood samples are analysed (Juul, Nielsen et al. 2012). Notably, results obtained in Denmark with saliva specimens were not quantitative when correlated with matching blood samples and it was discussed whether time of transport and storage conditions might have compromised enzyme availability in samples (Hede, Fjelstrup et al. 2018). All above mentioned factors have the potential to influence the sensitivity of saliva samples in malaria diagnostics and might have interfered with our method. Further studies will be required to extend the knowledge about the presence of parasite components in blood and other body fluids.

2.5 Climatic influences

There is evidence that humidity and high ambient temperatures have an impact on microscopes (Asp, Webber et al. 2020), test performance of malaria RDTs (Chiodini, Bowers et al. 2007) and the quality of dry blood spot samples on absorbent filter paper (Crimmins, Zhang et al. 2020). On one hand, it seems to suggest itself that the climatic differences between Denmark and Gabon can be widely disregarded, since the microfluidic assay at this stage was only performed in the air-conditioned laboratory and not yet outside of the laboratory buildings, where heat and humidity would be much more likely to influence the method. In the research laboratory of CERMEL, where the assay was performed, measures are taken to control certain conditions. Due to permanent air conditioning, the room temperature in all laboratory processing rooms is kept reasonably stable at an average of 22-25°C, which corresponds to the temperatures measured in the Danish laboratory. Indoor humidity control, on the other hand, is more difficult to maintain in tropical climate, despite the fact that ambient humidity inside is constantly reduced as a result of permanent air conditioning. During spot checks, the average humidity in laboratory processing rooms in Gabon ranged from 34% to 40%, which is only slightly higher than average ambient humidity in Europe of around 30%. Regarding the microfluidic assay, most of the material and equipment as well as the procedures themselves are considered not to be affected by heat and/or humidity. We can think of only one object whose functionality might be influenced with elevated humidity, which is the DNA column with its filter. Stored in the laboratory processing room in a sealed plastic bag, it should be resistant to the higher ambient humidity. Furthermore, the desired signal strength could be observed on the filters with positive controls, which reassured us and supported our hypothesis. Nevertheless, its functionality was not retested at any point during the process of troubleshooting. Further investigations regarding the impact of the different climate at the technology receiving site were not conducted due to the complexity of their design and conduction.

2.6 Questions that remain yet unanswered

Despite the known limitations we faced regarding the evaluated method, we cannot fully explain the results of the experiment shown on figure 23. The filters are coloured in different intensities, but they do not match the positive and negative microscopy results. For example, samples #29, #30 and #31 are microscopically negative for malaria infections, while in the assay at least sample #30 appears clearly as positive. On the other hand, sample #25 is microscopically positive. It remains unclear how to interpret the results obtained from this experiment, since an explanation for the mismatch between positives and negatives on the level of the experiment could not be found. It was considered and excluded to the highest extent possible that a mistake might have occurred when matching the saliva samples with the microscopy slides, nevertheless, definitive exclusion of such a possibility remains elusive in retrospect.

Not only questions about the microfluidic assay itself remain unanswered at this point, but also uncertainties about saliva as relatively new diagnostic medium in malaria diagnostics persist. So far, no standardized collection method for saliva samples has been established, therefore inconsistencies within analyses arise. Saliva can be collected unstimulated of, if larger quantities are required, stimulated through gustatory stimulation, chewing, citric acid or mastication. It has been described that saliva components such as bacteria, electrolytes or antibodies vary in their quantity depending on the saliva collection method (Ali, Starck et al. 2020), (Al Habobe, Haverkort et al. 2024), (Gomar-Vercher, Simón-Soro et al. 2018), however a more favourable collection method seems to remain subject to further research. In addition, food intake prior to collection might stimulate or inhibit specific elements in the saliva and individual mouth hygiene procedures are likely to alter the composition and characteristics of saliva. However, until now only little is known about the different influences on salivary components and its utilization in research and diagnostics. Other details, such as the fact that particular substances found in the saliva follow a circadian rhythm, are well described, but this has not yet transformed into a standard procedure for collecting saliva samples (Nunes, Mussavira et al. 2015). With the aim of creating

reproducible, reliable and generalized results with the microfluidic assay that can be compared inter- and intra-individually, i.e. between individuals (geographically, for example) as well as comparing the results of one individual over time, a standardized saliva collection method is essential. Factors influencing the saliva in any way have to be minimized to the largest possible extend in order to collect high quality data.

Summarising recent research concerning malaria diagnostics, two major goals can be identified. Firstly, methods capable of detecting very low parasite densities are needed in regions close to malaria elimination (Mbanefo and Kumar 2020). In these regions malaria prevalence is low and their predominant patient collective presents with low parasitaemia and is often clinically asymptomatic. Secondly, non-invasive sampling would bring further advantages in terms of patient comfort and compliance as well as a reduction of risks for health care workers due to lower infectivity (Owusu, Campillo et al. 2021). In addition, mass screening of patients would be more feasible when non-invasive sampling methods are applied. Furthermore, diagnostic methods ought to require minimal human resources and consumables, limited training of laboratory personnel and allow inexpensive purchasing to be applicable in low-resource settings. These requisites for ideal diagnostic tests in low-resource settings were coined by the WHO ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, delivered to those who need it) criteria (Mabey, Peeling et al. 2004). In the context considered, some criteria partially align with our methodology, while other criteria cannot be reliably assessed at this stage of development of the novel diagnostic assay and constitute subjects for future studies.

3. Limitations and weaknesses of this study

As described above, malaria diagnostics were performed by use of a rapid diagnostic test and thick and thin blood smear microscopy. The microscopy

results were used as comparator for the assessment and validation of the microfluidic assay. As no PCR was performed, potential sub-microscopic parasitaemia might have been missed, however from a clinical perspective microscopy is the gold standard diagnostic. Modern molecular parasite diagnostic methods have a particularly low detection limit of <10 parasites per microliter. Sub-microscopic malaria infections might explain why certain saliva assays turned positive while the thick smear microscopy was read negative as for even expert microscopy a detection limit of around 10 parasites/µl blood is described. But as the detection limit for the microfluidic assay is still to be determined and it is unclear whether the amount of malaria parasites in saliva is directly associated with the parasitaemia found in blood, this is open to speculation.

Saliva was collected unstimulated in a spontaneous way, which means patients were asked to spit into a collection cup without any further stimulation of the saliva production or preparations prior to sampling. Especially younger children often had difficulties to collect the saliva in their mouth before transferring it into the cup, while adults could mostly spontaneously provide a sample without any inconvenience. This study was a pilot study investigating the prospects of saliva as diagnostic specimen. No specific information about the collection of saliva was recorded, a fact which is by hindsight a limitation of our work. Information on dental hygiene status and standards, last food and drink intake and previous or current infections in the mouth region could have been assessed and an inspection of the oral cavity, gingiva, teeth and tongue could have been included in the sample collection process. This information could have contributed to extend the knowledge about sample quality and constitution and the thereby affected accuracy of obtained results.

Moreover, the impact of the different climatic zones between the countries of the transferring and the receiving laboratory was not further investigated in detail. A possibility to test the functionality of the used DNA columns could have been realized by sending some of these consumables back to Denmark to compare the influence of storing and shipment conditions in Gabon to those in Denmark.

Another weakness of the here described process of systematic evaluation of the implementation of the diagnostic test was the fact that optimisations and adaptions were done on an ad-hoc basis due to the previous successful proof-of-principle phase in Denmark. Therefore, the modifications during this phase of the project could not always be executed in an evidence-based approach, which all in all led to a fairly time and resource consuming process compared to the final findings.

4. Summary of accomplishments and further recommendations

The purpose of this test implementation and optimisation project was to advance the implementation of the microfluidic assay for malaria diagnostics from human saliva to Gabon, where the further evaluation of the method was planned. We aimed to optimise the assay in a way to eventually produce reliable results, where negative and positive samples can be clearly distinguished and which match the comparative microscopic blood smear analyses. This here described assessment contributed to gain important information, which serves as a basis for further investigations. We were able to verify the quality and consistency of certain reagents and thereby prove their functionality. Furthermore, the cross-contamination onto subsequent samples could be minimized and a clearer negative control could be obtained due to a new, adapted protocol. It was also shown that the filters can generally result in high colour intensities as seen on the positive controls. Moreover, different steps of the method were investigated, modified and improved or proven efficient.

According to all the optimisations and improvements that were investigated and approved during this project, a SOP was elaborated. The SOP, which assembles the changes that were made in the original protocol in favour to obtain the most advanced version shown in the summary of results, can be used as a guiding document. It provides detailed listings of required materials, background information and explanations regarding the method and step-by-step instructions

to allow the reader to conduct the entire microfluidic assay completely independently. The here described SOP can be found in the Annex of this work. The SOP is the summary and the outcome of the process of systematic assessment of the test implementation described in this work and builds the basis for further optimisations of the assay. Despite all the efforts that were undertaken, ideas that were followed-up and optimisations that were put into effect, we could in the end not achieve a successful implementation of the assay in Gabon. This is most likely due to the still premature stage of development of the microfluidic assay, which seemed not yet appropriate for testing of clinical samples. We have to assume that the method is not yet sufficiently developed for the intended set up, so that the optimisation steps tested in this work are not sufficient to improve the overall outcome to an extent that allows successful test implementation. We are convinced that more systematic and fundamental analyses are required to reveal causes of failure and implement new processes within the method in order to accomplish the desired optimisation and meet the obligatory requirements needed to fully implement the assay. The proof-of-principle was successful, yet we recommend to further systematically investigate the mechanisms of this new method to gain a better understanding of the single steps and to enable more deliberate modifications and optimisations of the assay. Also, statistical parameters such as detection limits, sensitivity and specificity and positive and negative predictive value were not yet evaluated and evidently imply further research on these topics. These information need to be obtained so that a precise interpretation of the assay is ensured and its results can be used accordingly to treat patients in the best and most appropriate way possible. In the interim, the assay development has been advanced in Denmark based on our results and a refined experimental protocol developed. A reevaluation of the test in Gabon, as well as its implementation, is currently in the planning stage by an international academic consortium.

V Conclusion

Since the start of the new millennium international funding for malaria control was increased about twentyfold. Control strategies were set in place and estimations reveal that around 663 million symptomatic malaria cases were averted in Sub-Saharan Africa between 2000 and 2015 (Bhatt, Weiss et al. 2015). Moreover, within 15 years, 17 countries eliminated malaria and the worldwide malaria mortality decreased by 62% since the turn of the century (WHO 2016a).

These facts and numbers show the important success in the process of malaria control towards the vision of eventual malaria elimination and ultimate malaria eradication. However, new challenges are constantly arising and it is evident that the battle against malaria is not yet won. Greenwood (2017) states that various circumstances will even rather impede the ongoing process of elimination of malaria during the coming years (Greenwood 2017).To keep up political stability and the necessary infrastructure in areas of high malaria transmission in order to successfully implement and maintain disease control standards will be only one difficulty. Also maintaining the funding for malaria at a similarly high level as given over the past years is a challenge to face, especially in periods of political instability and when incidence is declining and a considerable progress has already been made. In addition, as already mentioned earlier, the emergence of drug resistant *Plasmodium* strains complicates treatment and as well resistances of mosquitos to insecticides lower the effectiveness of bed nets and might influence success of prevention measures in the future. The fear that malaria incidence and mortality might increase again due to the above-mentioned factors seems possible according to Greenwood's report (Greenwood 2017). On the other hand, lessons learned from the emerging drug resistance in Asia made researchers and clinicians more aware of the importance of constant investigation and assessment of new mechanisms of action for future antimalarial drugs (Antony and Parija 2016). Furthermore, due to the development of new

technologies during the past years it is now possible to monitor drug resistance much more effectively than before.

It has been shown that diagnostics make a major contribution to successful malaria control by providing a basis for correct treatment, avoiding an overuse of antimalarials while detecting those who carry a current malaria infection (Peeling, Smith et al. 2006), (Zimmerman and Howes 2015), (2011), (Leslie, Mikhail et al. 2012). Furthermore, diagnostic methods help to monitor the epidemiological characteristics of *Plasmodium* species and subsidise disease surveillance. Despite their important role in malaria control, thick smear microscopy and rapid diagnostic tests alone are not sufficient to achieve the elimination of malaria. To set an end to malaria, more precise and easier to use diagnostic techniques are required. Moreover, non-invasive sampling, a minimal complexity and stable performance under various conditions and cost-effectiveness are further required characteristics for malaria diagnostic methods if they aim to importantly support malaria elimination efforts (Zimmerman and Howes 2015).

The droplet microfluidic assay for malaria diagnostics from human saliva is a new method of diagnosing malaria infections. By analysing saliva to detect the presence of infections, it holds a pioneering role and the promise of revolutionizing malaria diagnostics. The here described new test has passed the proof-of-principle phase when performed in Denmark before its pilot implementation in Gabon.

In this work, a systematic process of optimisations and assessment of limitations of the microfluidic assay is described and discussed. Although the desired implementation of the test in a large prospective study could not be achieved during the course of this project due to the persistent limitations in diagnostic accuracy, the improvements that were made and the adaptions, which were investigated during this project, will enable the further development of the here presented assay. The importance of and the need for new diagnostic methods for malaria is evident and once successfully optimised, implemented and evaluated, the droplet microfluidic assay has the potential to importantly contribute to malaria elimination efforts.

VI Summary

Background

Malaria is a potentially life-threatening parasitic disease transmitted by the bites of the female anopheles mosquito.

In 2022, 249 million malaria cases were reported worldwide, 608000 of them with deadly outcome. The highest burden is carried by the African continent, were more than 90% of both global cases and malaria related deaths occurred in the past decades. As estimated by the WHO, half of the world's population was at risk for malaria in 2022.

Testing strategies play an important role in the process of malaria elimination. Established diagnostic methods are based on blood specimens. Obtaining these samples poses an operational limitation due to its invasiveness and potential hazards. In Denmark a new and innovative enzyme-based methodology was proven to successfully detect malaria parasites in human saliva. This work describes the process of technology transfer of the newly developed method to a malaria endemic region and the systematic implementation in a pilot study and the assessment of its technical limitations. The aim of the pilot study was to adapt the assay to the different environment and to elaborate a SOP for further implementation of the test.

Material and methods

The pilot study was performed at the Centre de Recherches Médicales de Lambaréné, CERMEL, in the central African country Gabon. 43 patients with suspected malaria consented to provide paired blood and saliva specimens. Blood samples were analysed using rapid diagnostic tests and blood smear microscopy to confirm or rule out malaria infections. Saliva samples were examined with the new method and compared to the microscopy results. The principle of the so-called REEAD (rolling circle amplification enhanced enzyme activity detection) method is the visualization of a malaria parasite specific enzyme due to its ability to alter DNA shape. To achieve hand driven enzyme

extraction, mechanical stress is applied via a microchip to lyse the parasite and release the target enzyme, a topoisomerase I. The following DNA amplification step can only take place if the target enzyme is present. Therefore, hairpin shaped DNA fragments are added and due to the cleavage function of the topoisomerase closed DNA rings are formed. After hybridization with a primer, a polymerase is able to amplify the DNA template. Without the target enzyme i.e. without a malaria infection, no DNA rings are being formed and amplification cannot take place. For later visualization biotin-linked nucleotides are used in the amplification step. During the last step, HRP, a peroxidase, binds to biotin on a filter membrane and catalyses the oxidation of TMB (tetramethylbenzidine) to a blue end product. Eventually, a colorimetric readout of the filter membranes can be done visually. Malaria positive saliva samples result in blue filter membranes while negative samples remain uncoloured.

Results and discussion

With the implementation of the first set of assays in Gabon it became evident that positive samples resulted in a weak blue signal and negative samples likewise appeared blue leaving limited potential to discern true positive samples from negative specimens. Furthermore, the second negative control consistently resulted in a blue stain. Based on these factors, the distinction between positive and negative samples was insufficient when the test was implemented in Gabon. A process optimisation study was performed to rule out potential contamination and improve test performance by improving the test procedures.

In paired tests performed in Gabon and Denmark, overall signal intensity in Gabon remained weak and a reduced sample stability at similar storing conditions was observed. Integrity of both reagents and the polymerase strains was assured and alterations of DNA substrate and polymerase concentrations did not increase signal intensity without increasing signal intensity in negative controls at the same time. Overall, the process optimisation study was able to rule out intrinsic test factors as reason for the lower-than-expected test performance.

It remains questionable whether climatic conditions in Gabon or during the shipment unduly influenced the filter membrane's features and therefore might have had a negative impact on the outcome of the assay. Standardized saliva sampling as well as the performance of PCR on blood samples in order to detect sub-microscopic malaria infections may provide further insights.

Conclusion

The droplet microfluidic assay for diagnostics of malaria infections is based on saliva analysis and has shown to be a promising approach at the phase of proof-of-principle in Denmark. During the pilot-study for technology transfer to Gabon this new method was adapted to the local conditions and a SOP was elaborated. Compared to blood smear microscopy - the gold standard diagnostic tool for malaria - the REEAD method was demonstrated to yield poorer diagnostic performance in this setting while the reasons for this still remain unclear.

The need for new diagnostic methods to eliminate malaria worldwide is undeniable. Affordable, non-invasive, sensitive testing adapted to low-resource-settings is required to identify both symptomatic and asymptomatic malaria carriers in order to interrupt transmission. Implementing saliva-based methods bear the potential to accelerate malaria elimination efforts particularly in population-based surveys and control programs. The elaborated SOP may serve as a groundwork for further optimisations of the described assay. Findings gained from the experiments possibly support the development of other saliva based diagnostic methods in order to reach the vision of a world free from malaria by 2030.

VII Zusammenfassung

Einleitung

Malaria ist eine potentiell lebensbedrohliche parasitäre Erkrankung, die durch Stechmücken übertragen wird.

Im Jahr 2022 wurden weltweit 249 Millionen Malariainfetionen registriert, von den 608.000 Fälle tödlich endeten. Besonders betroffen ist der afrikanische Kontinent, wo sich in den vergangenen Jahren über 90% der der Infektion und Todesfälle ereigneten. Die Weltgesundheitsorganisation schätzt, dass 2022 etwa die Hälfte der Welbevölkerung vom Risiko einer Malariainfektion betroffen war.

In der Bekämpfung der Malaria kommen diagnostischen Tests eine besondere Bedeutung zu. Herkömmliche Testmethoden basieren auf Blutproben, deren Gewinnung und Verarbeitung jedoch erhebliche Risiken bergen. In Dänemark gelang es mithilfe einer neuen Methode, Malariaparasiten zuverlässig in Speichel nachzuweisen. In der hier vorliegenden Arbeit wird der Prozess des Technologietransfers dieser Methode in ein malariaendemisches Land beschrieben. Ziel dieser Pilotstudie war es, den diagnostischen Test an die neuen Bedingungen anzupassen und zu optimieren, sowie eine SOP für die Durchführung des Tests zu erarbeiten.

Material und Methoden

Die Durchführung der Pilotstudie erfolgte am Centre de Recherches Médicales de Lambaréné, CERMEL, im zentralafrikanischen Gabun. Von 43 Patientinnen und Patienten mit vermuteter Malariainfektion wurden Blut- und Speichelproben gewonnen. Anhand von Malaria-Schnelltests und Mikroskopie wurde die Verdachtsdiagnose überprüft. Die Speichelproben wurden mit der zu implementierenden Methode analysiert und mit den Ergebnissen der mikroskopischen Untersuchung des Blutes verglichen. Für die sogenannte REEAD-Methode (rolling circle amplification enhanced enzyme activity detection) wird ein aktives Enzym der Malariaparasiten, die Topoisomerase I, manuell aus den Speichelproben extrahiert. Dies erfolgt mithilfe eines Mikrochips, in welchem

die Parasiten durch mechanischen Stress lysiert werden und das Zielenzym freigesetzt wird. Anschließend folgt eine DNA-Amplifikation, welche nur in Anwesenheit des extrahierten Enzyms erfolgen kann. Dafür werden haarpinförmige DNA-Fragmente hinzugegeben, welche durch die Topoisomerase zu Ringen geschlossen und nach Hybridisierung mit einem Primer durch eine Polymerase amplifiziert werden. Enthält die Speichelprobe keine Malariaparasiten, kann der DNA-Strang nicht geschlossen und dadurch nicht vervielfältigt werden. Zur späteren Visualisierung des DNA-Produkts werden biotinmarkierte Nukleotide für die Amplifizierung verwendet. Im letzten Schritt bindet HRP, eine Peroxidase, auf der Filtermembran an Biotin und katalysiert die Oxidation von chromogenem TMB (Tetramethybenzidin) zu einem blauen Endprodukt. Schließlich kann das Ergebnis auf einem Filter kolorimetrisch abgelesen werden. Dabei färben sich die Filtermembranen malariapositiver Proben blau, während Filter der Proben ohne Malariaparasiten weiß bleiben.

Ergebnisse und Diskussion

Nach der ersten Implementierung des Assays in Gabun zeigte sich, dass positive Proben teilweise nur schwach blau gefärbt erschienen, während sich bei negativen Proben ebenso eine dezente Färbung darstellte. Zudem zeigte sich eine leichte Blaufärbung der zweiten Negativkontrolle. Dadurch ließen sich positive von negativen Proben unzureichend unterscheiden. Eine Kontamination des verwendeten Materials durch zuvor verarbeitete positive Proben wurde vermutet, sodass Zwischenschritte mit aggressiveren Reinigungsmethoden eingeführt wurden.

Im Vergleich zu den in Dänemark durchgeführten Experimenten zeigte sich außerdem eine deutlich geringere Intensität des Farbsignals auf den Filtern sowie bei gleicher Lagerung eine erheblich geringere Probenstabilität. Verschiedene Experimente bestätigten die Funktionalität der Reagenzien und verwendeten Polymerase-Batches. Durch die Veränderung von Konzentrationen verschiedener Reagenzien konnte keine Optimierung der Ergebnisse erzielt

werden aber intrinsische Probleme des Testmaterials und des Prozesses ausgeschlossen werden.
Unklar bleibt, ob die klimatischen Bedingungen in Gabun die Eigenschaften der Filtermembran beeinflussen. Eine standardisierte Gewinnung von Speichelproben sowie die Durchführung einer PCR zur Diagnostik submikroskopischer Malariainfektionen könnten in zukünftigen Experimenten weitere Erkenntnisse erbringen.

Schlussfolgerung

Der in dieser Pilotstudie untersuchte Assay erbrachte in der Phase des proof-of-principle in Dänemark vielversprechende Ergebnisse. Im Rahmen der nach dem Technologietransfer nach Gabun durchgeführten Pilotstudie konnte die Leistung des Diagnostiktests unter den neuen Bedingungen optimiert und eine SOP erarbeitet werden. Verglichen mit der Referenzdiagnostik durch Blutmikroskopie bleibt die sogenannte REEAD-Methode jedoch zum aktuellen Zeitpunkt der Standarddiagnostik deutlich unterlegen.
Die Dringlichkeit neuer diagnostischer Methoden zur weltweiten Elimination der Malaria ist unumstritten. Kostengünstige, nicht-invasive und sensitive, an ressourcenarme Regionen angepasste Testmaßnahmen sind notwendig, um symptomatische sowie asymptomatische malariainfizierte Träger schnell zu identifizieren. Nur so kann die Übertragung langfristig unterbrochen werden. Die Implementation eines auf Speichel basierenden Tests, der die genannten Kriterien erfüllt, hätte das Potential, die Malariaelimination maßgeblich voranzutreiben. Die durch diese Pilotstudie erarbeitete SOP kann als Grundlage für weitere Optimierungen des beschriebenen Tests dienen. Ergänzend können die in den Experimenten gewonnenen Erkenntnisse die Entwicklung neuer speichelbasierter Testmethoden unterstützen, um der Vision einer Welt frei von Malaria bis zum Jahr 2030 ein Stück näher zu kommen.

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IX Contributions and Declaration of Originality

Birgitta R. Knudsen and her team from Aarhus University, Denmark, developed the microfluidic assay for the detection of malaria parasites in human saliva. She arranged the technology transfer to Gabon and supervised the experiments performed in Gabon and their interpretation along with the planning of further experiments.

Prof. Dr. Michael Ramharter, formerly Medical University of Vienna, Austria, and Centre des Recherches Médicales de Lambaréné CERMEL, Gabon, coordinated and supervised the overall project. Prof. Dr. Ramharter furthermore supervised and corrected this manuscript.

Dr. Ghyslain Mombo-Ngoma, head of the Clinical Operations Department at CERMEL, Gabon, led the coordination and conduction of the project on site. He and his team organised the collection of samples and arranged patient care.

Input and ideas for troubleshooting, modifications and new experiments on site were mainly given by Dr. Marguerite Massinga Loembe, head of the research laboratory at CERMEL, Dr. Matthew McCall and Rodrigue Bikangui. Dr. Marguerite Massinga Loembe also assisted in tracing documents on public health data in Gabon and developing a structure for this work.

Thick smear microscopy was performed by microscopists of the clinical laboratory of CERMEL and by myself. All experiments described in this work, with exception of those carried out in Denmark, I performed and documented by myself. I have documented all methods, data and processes truthfully and I have not manipulated any data. I independently completed the statistical analysis using and performed the measurements of filter intensities and filter brightness as well as correlation analyses between filter colour intensities of saliva samples and corresponding microscopic thick blood smear parasitaemia by myself. I executed the literature review with no other sources than listed.

Contributions and Declaration of Originality

I confirm that I am the sole author of the written work here enclosed and that I have compiled it in my own words. Parts excepted are corrections of form and content by the supervisor.

Tübingen, January 29th, 2024

Ronja Neher

X Publication note

Parts of this work have already been published:

Hede, M. S., Fjelstrup, S., Lötsch, F., Zoleko, R. M., Klicpera, A., Groger, M., Mischlinger, J., Endame, L., Veletzky, L., Neher, R., Simonsen, A., Petersen, E., Mombo-Ngoma, G., Stougaard, M., Ho, Y. P., Labouriau, R., Ramharter, M., & Knudsen, B. R. (2018). Detection of the Malaria causing Plasmodium Parasite in Saliva from Infected Patients using Topoisomerase I Activity as a Biomarker. *Scientific reports*, *8*(1), 4122. https://doi.org/10.1038/s41598-018-22378-7

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Annex 1 Sample Log

Sample #	Parasitaemia in thick smear	RDT	Age
1	753 p/µl	+	5 years old
2	2166 p/µl	+	48 y/o
3	1350 p/µl	+	7 y/o
4	789 p/µl	+	11 y/o
5	6869 p/µl	+	5 y/o
6	962 p/µl	+	26 y/o
7	2739 p/µl	+	4 y/o
8	437 p/µl	+	8 y/o
9	47 p/µl	+	14 y/o
10	182 p/µl	+	Children < 10 y/o
11	285 p/µl	+	
12	496 p/µl	+	
13	679 p/µl (+1 Gametocyte)	+	
14	1517 p/µl	+	
15	2403 p/µl	+	
16	3873 p/µl	+	
17	4480 p/µl	+	
18	32270 p/µl	+	
19	3791 p/µl	+	7 y/o
20	6755 p/µl	+	4 y/o
21	2793 p/µl	+	5 y/o
22	Neg.	+	42 y/o
23	Neg.	-	3 y/o
24	Neg.	+	5 y/o
25	188100 p/µl	+	5 y/o
26	Neg.	-	5 y/o
27	2800 p/µl	+	8 y/o
28	180000 p/µl	+	2 y/o
29	Neg.	-	53 y/o
30	Neg.	-	7 y/o
31	Neg.	+	53 y/o
32	9113 p/µl (Pf+Po)	+	11 y/o
33	Neg.	+	9 y/o
34	Neg.	-	9 y/o

Sample #	Parasitaemia in thick smear	RDT	Age
35	Neg.	+	5 y/o
36	Neg	+	30 y/o
37	Neg.	+	6 y/o
38	14307 p/µl	+	23 y/o
39	148 p/µl	+	6 y/o
40	Neg.	-	24 y/o
41	33959 p/µl	+	7 y/o
42	2081 p/µl	+	2 y/o
43	Neg.	-	27 y/o

Annex 2 SOP

STANDARD OPERATING PROCEDURE

Title	TopoisomeraseI Microfluidic Assay for Malaria Detection in Human Saliva
Short title	TOPOMAL2 Saliva Assay

OBJECTIVE

The objective of this SOP is to describe the procedure of the topoisomerase I microfluidic assay which aims to detect the malaria parasite plasmodium using human saliva as test material. The procedure is a part of the study TOPOMAL2.

APPLICATION/SCOPE

This SOP is applicable to the following departments/core job descriptions of CERMEL:

Laboratory technicians and researchers who are involved in TOPOMAL2 and perform the assay described in this SOP in the research laboratory

DEFINITIONS AND ABBREVIATIONS

Definitions : N/A

Abbreviations :

CERMEL Centre de Recherches Médicales de Lambaréné

SOP Standard Operating Procedure

RESPONSIBILITIES

Staff	Task
Researchers involved in TOPOMAL performing the assay	Read, understand and be able to apply this SOP in the research laboratory
Laboratory supervisor	Ensure that this SOP is implemented as described

PRINCIPLE

The topoisomerase I microfluidic assay consists of the following steps:

Lysis of the malaria parasite and extraction of the target enzyme (pfTopoI)

The plasmodium expressed enzyme, topoisomerase I (pfTopoI) is extracted from human saliva via *mechanical stress* combined with a hypotonic lysis buffer. Extraction is achieved using a microfluidic chip with a serpentine channel and frees pfTopoI to convert a DNA substrate into a detectable DNA product.

Rolling circle amplification of a DNA substrate by the topoisomerase I

The target enzyme pfTopoI transforms a non-circular DNA substrate into a covalently closed DNA circle. The generated DNA circle is amplified via a *rolling circle amplification* reaction (RCA). Biotin-linked nucleotides are incorporated into the RCA product to allow subsequent visualization of the generated RCA product.

Visualization of the amplified DNA circles

The RCA products are bound to a silica membrane. Streptavidin conjugated HRP (horseradish peroxidase) is bound to the biotinylated RCA products and can be visualized by exploiting an oxidation reaction carried out by HRP, where the HRP substrate TMB is converted into a blue product, which can be seen on the silica membrane.

Readout

Saliva samples from malaria patients are expected to result in blue membranes whereas saliva from uninfected individuals are expected to result in white membranes.

Detection limits, specificity and sensitivity are to be found out during the study.

SAFETY AND ENVIRONMENT

Follow general biosafety precautions applicable to a BSL-1 laboratory, notably:

Wear protective gloves and a lab coat.
Do not eat and drink in the laboratory.
Clean and disinfect all spills of specimens or reagents using 70% Ethanol.

SAMPLES

The required sample is a saliva sample. Minimum sample amount: 0,5 ml. The saliva is supposed to be transported at 4°C and stored in a fridge at 3-7°C. Saliva samples can also be frozen at -20°C, but once thawed, samples cannot be refrozen again.
The maximal storage duration for samples which are kept in the fridge is to be determined within the study.

EQUIPMENT AND SUPPLIES

Reagents and Chemicals

Oil (2% Pico-Surf (TM) 1 in Novec 7500; Dolomite- microfluidics, 3200215)
PBS (Dulbecco's Phosphate Buffered Saline; Sigma, D8537)
Tris-HCl
DTT (Dithiothreitol)
Tween 20
PMSF (Henylmethylsulfonyl fluoride)
DNA substrate (5'-TCTAGAAAGTATAGGAACTTCGAACGACTCAGAATGACTGTGAAGATCGCTTATCCTCAATGCACATGTTTGGCTCCCATTCTGAGTCGTTCGAAGTTCCTATACTTT-3')
EtOH 95%
Milk powder
dH_20
BSA (bovine serum albumin)
dNTP (1mM of each of the nucleotides dATP, dCTP, dGTP and dTTP. 10% of dCTP was substituted with Biotin-16-Aminoallyl-2'-deoxycytidine-5'-Triphosphate from Trilink Biotechnologies)

Primer 1 (5'-CCT CAA TGC ACA TGT TTG GCT CC)

Primer 2 (5'- CCA AAT AAG CGA TCT TCA CAG T)

Phi29 buffer supplied with the Phi29 enzyme

Phi29 polymerase (ThermoFisher Scientific, EP0094)

TE-buffer (Ethylenediaminetetraacetric acid; 10 mM Tris-HCl pH 7,5; 1 mM EDTA)

Urea powder

Binding buffer CP (Omega Biotek PDR042)

HRP (Pierce Streptavidin Poly-HR, 21140)

TMB (TMB Enhanced One Component HRP Membrane Substrate; Surmodics, ESPM-0100-01)

Drop release agent (1H,1H,2H,2H-Perflouro-1-octanol; Sigma 370533-25G)

Equipment and Material

Microfluid chip

Outlet tubing (VWR, HAMI20924)

Inlet tubing (VWR, HAMI90624)

Syringes 1ml (Abena, 22801201)

Eppendorf tubes 1,5ml

200µl tubes

500µl tubes

Parafilm

DNA columns

(Omega Biotek, DNACOL2-01)

- P2 pipette + tips
- P20 pipette + tips
- P200 pipette + tips
- P1000 pipette + tips
- Transparent file
- Glue
- Cotton tips
- Blank paper
- Paper scanner

- Vortex
- Table centrifuge
- Scissors

QUALITY CONTROL

Negative and positive controls are used to ensure the single steps of the procedure are done correctly and furthermore they can provide information in case of troubleshooting.

Control type	Sample details	Explanation
Negative Control	True negative control saliva brought from Denmark	Shows that there is no contamination of reagents or material
Positive Control	Sample tested positive before	Shows that extraction of the parasite, amplification and readout of samples are done correctly
	Sample containing produced DNA circles (end product)	Shows that readout is done correctly
	Sample containing DNA substrate and the purified target enzyme pfTopoI	Shows that the DNA substrate is working

The control samples are processed in the following order: negative control → samples to be analysed → positive control → negative control
If the controls show different results than expected, the assay will be repeated.

PROCEDURE

Preparation of the microfluidic chip

1. Attach the oil inlet tubing (max. 4 cm) to the syringe. Fill the syringe with oil and remove air from the syringe and the attached oil tubing
2. Attach the oil inlet tubing attached to the oil-filled syringe to the microfluidic chip. Attach the outlet tubing (max. 4 cm) to the microfluidic chip.
3. Fill the chip with oil using a syringe and leave for 15 minutes. The oil provides a hydrophobic channel system in the chip.

In the meantime, prepare the sample.

Lysis of the malaria parasite

4. Spin the saliva sample (15µl) through a 500 µl tube with a hole in the bottom into a 1,5 ml micro centrifuge tube. This homogenizes the saliva.

5. Dilute 10µl homogenized saliva by mixing it with 490 µl saliva dilution buffer (PBS). Invert the tube gently.

Remember to include two negative and one positive control.

6. Mix 30 µl diluted saliva with 60 µl of lysis buffer supplemented with DNA substrate. Add 180 µl oil to the mixture.

Preparation of the lysis buffer:

Mix 10mM Tris-HCl (pH 7,5); 5 mM EDTA; 1mM DTT; 0,2 V% Tween
Just before use, add 1 µl DNA substrate to 400 µl lysis buffer
Add 0,1 V% PMSF

The lysis buffer can kept for a week in the fridge. It is hypotonic in order to lyse the parasites.

7. Vortex the mixture for 1 minute.

8. Pull all of the generated emulsion into a clean syringe via an attached sample inlet tubing (max. 4 cm).

9. Remove the oil-filled syringe with the attached tubing from the chip and attach the tubing with the emulsion-filled syringe to the chip.

10. Slowly press all of the emulsion through the microfluidic channel and keep the tip of the syringe pointing upwards during this movement.

11. Collect all of the emulsion dripping from the outlet tubing in a parafilm coated 200 µl tube.

Incubate the droplets overnight.

12. Remove the syringe with the inlet tubing from the chip. Discard the used chip and the outlet tubing

For processing the next sample, wash the inlet tubing in the following way:

Pull 5x dH20 into the tubing and attached syringe

Change the syringe

Again, pull 5x dH20 into the tubing and the newly attached syringe

Pull 5x EtOH into the tubing in syringe
Change the syringe
Rinse the tubing and the syringe with 2x 200 µl wash oil
The inlet tubing and the syringe are now prepared for step 8.

13. Continue now with step 1 to prepare the next microfluidic chip with oil. Follow then steps 4 to 12 until all the samples including the negative controls are processed in this way.
14. The next day, transfer the incubated droplets into an Eppendorf tube.
15. Break the droplets by adding 25 µl drop release agent (1H, 1h, 2H, 2H perfluoro-1-octanol).
16. Mix the content of the tube and wait 30 seconds and spin in the table centrifuge. Check that the tube contains two phases and that the upper phase is clear. Collect the upper phase and move it into a 200 µl tube.
17. Keep 3 µl for the following step. Aliquot the remaining circles and freeze them in two different tubes at -20°C. One tube is for shipping to Denmark, the other one is kept on-site as a back-up.

Rolling circle amplification

18. For each experiment include a positive control with containing DNA circles.
19. Number of samples (including controls) to be analysed is denoted n. Make a mastermix sufficient for n+1 reactions (or +10% of n if more than 10 samples are tested). Mix everything expect the Phi29polymerase. Then add the Phi29polymerase and mix again by pipetting.

Preparation of the mastermix:

Reagent:	µl used for 1 reaction	Stock concentration
BSA	1	10 mg/mL
dNTP	1	1 mM each nucleotide (10% of dCTP is conjugated with biotin)
Primer 1	0,5	100 µM
Primer 2	0,5	100 µM
Phi29 buffer	1	10X
Water	2	-
Phi29 polymerase	2	10 U/µl

20. Mix 2 µl of each of the samples from 16 or the control circles with 8 µl of the master mix.
Incubate for 1 hour at 37°C.

Readout

21. Add 90 µl TE buffer to each of the samples.
22. Add 400 µl binding buffer (CP) the each of the samples.
23. Add the mixture to a spin column. Incubate for 5 minutes, spin (spin: centrifuge for 5 seconds in a table centrifuge).
24. Add 200 µl PBS, spin. Repeat.
25. Add 200 µl blocking buffer to the spin column, incubate for 5 minutes, spin.

Preparation of blocking buffer: Dissolve 0,5 g milk powder in 10mM Tris-HCl pH 7,5 to a total volume of 10 mL.

Can be kept in the fridge for 3-4 days.

26. Add 200 µl PBS, spin. Repeat.
27. Add 200 µl urea solution to the spin column. Incubate for 5 minutes. Spin.

Preparation of urea solution: Dissolve 4,8g Urea in dH_20 to a total volume of 10 mL.
Can be kept in the fridge for 2 weeks.

28. Add 200 µl PBS, spin. Repeat.
29. To prepare visualization mixture for 1 sample, mix 1,2 µl HRP with 150 µl blocking buffer. Make visualization mixture for n+1 samples (or n+10% when more than 10 samples are tested). 30. Mix by gently pipetting.
31. Add 150 µl visualization mixture to each column, incubate for 10 minutes, spin.
32. Add 150 µl PBS supplemented with 0,2V% Tritin-x100, spin. Perform this step a total of 3 times.
33. Add 150 µl TMB. Positive samples will become blue. Colour development usually takes 5-10 minutes.
34. When the desired signal strength is reached, spin and stop the reaction by adding 200 µl water and spin again.
35. For quantification and documentation remove the filters from the column and attach to plastic using glue.

INTERPRETATION, APPROVAL, AND RELEASE OF RESULTS

Processing the results

The filters which are attached to the plastic are scanned in a paper scanner and in addition a (colour) copy is made. The digital results are quantified using e.g. ImageJ.
The copy is kept with the sample processing sheet in a binder labelled “TOPOMAL2”.

REFERENCES

ISO. Medical laboratories – Requirements for quality and competence. ISO 15189. Geneva, Switzerland: International

ASSOCIATED DOCUMENTS

Records

- a-bb-cc-Rc (Vdd): Title

SOP`s

- a-bb-cc-Vdd-ee: Title

Manuals

- Quality Manual - located in the Quality Manager´s Office

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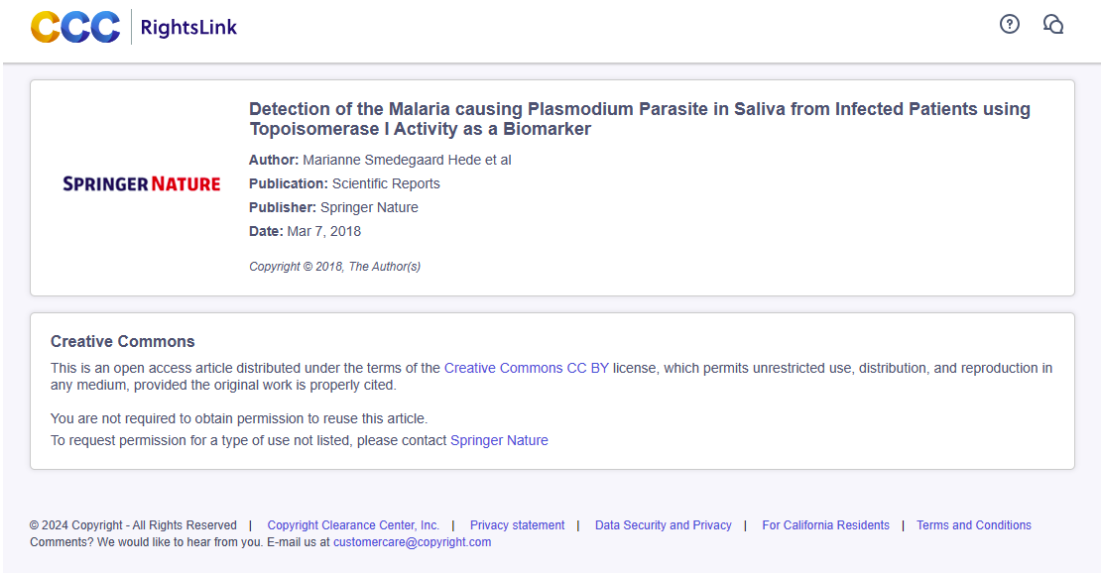

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Author: Marianne Smedegaard Hede et al
Publication: Scientific Reports
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