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IMPLEMENTATION OF A MULTIPLEX ASSAY FOR DETECTION OF ANTIBODIES AGAINST DIFFERENT (20) PLASMODIUM ANTIGENS:

A potential tool for evaluating anti-malarial interventions

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Master dissertation submitted in completion of the degree of
Master in Biomedical Sciences

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Contributions to this thesis

Sample collection of the survey has been performed by the CNM Phnom Penh (National Center for Parasitology, Entomology and Malaria Control)/ ITM Antwerp team in Ratanakiri province in Cambodia. The study design (Project Proposal) and the literature research done at the ITM is performed by myself under direct supervision of LD and corrected by MC. The protocols are written by myself, verified by LC and validated by DM. I performed all the practical work at the Institut Pasteur du Cambodge under the direct supervision of LC and LD. Selection of the pool of sera and the high positive control dilutions is done by me, LD and LC. Data entry and analysis of the results was done at the ITM by myself, except to define the limit of acceptance for high positive controls and the cut-off value with the preprogrammed STATA software and the figures 26, 27 and 28 belonging to paragraph 4.10 (made with the R-software), which was done by LD. I did the writing of the thesis that is corrected by LD and MC. This work is part of a larger project on the evaluation of large use of tropical repellent in addition to insecticide treated nets in the control of malaria transmission (Bill & Melinda Gates Foundation)

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Abstract

1. Introduction

Malaria is the most common mosquito-borne infection worldwide. The infection is caused by a parasite of the genus *Plasmodium*. In Cambodia about 2.1 million people, of whom 500.000 live in the high-endemic forested regions, are at risk. Cambodia is characterised by a low prevalence and incidence of malaria, and detection of parasitological indicators to estimate the transmission intensity can be difficult. Therefore, the aim of this master thesis is to implement a recently developed multiplex assay for the simultaneous detection of multiple antibodies (Abs) to ultimately detect the presence of malaria parasites in human blood samples. This work fits within the framework of a large-scale research project aimed to evaluate the effect of mass use of safe and effective mosquito repellents on the malaria transmission, in addition to the use of impregnated mosquito nets. One of the outcomes in this project is based on serology.

2. Methodology

An assay based on 14 *Plasmodium* specific peptides LSA1-41, LSA1-J, LSA3-NR2, LSA3-RE, GLURP, GLURP-P3, SALSA1, SALSA2, CSP, SR11.1, STARP-R, Pm CSP, Pv Like CSP and PvVK210 CSP, 1 peptide (SALIV2) specific for the *An. gambiae* saliva protein and 5 Plasmodium specific recombinant proteins Pf GLURP R2, Pf MSP1-19, Pv MSP1-19, Pv DBP and Pf13 was developed for the MAGPIX system and applied on blood spot samples from 1440 individuals from Ratanakiri province, Cambodia.
Implementation of the assay started with dissolving of the antigens (Ags), followed by a selection of the positive control sera by ELISA (17 random selected sera are tested on 24 Ags). Then, covalent coupling of the beads was first tested on 1 *Plasmodium* specific peptide (GLURP)in small scale ($1*10^6$ beads) followed by GLURP in large scale ($5*10^6$ beads) and then recombinant proteins ($1*10^6$ beads), after which the mass coupling of the beads was done on $5*10^6$ beads. Furthermore, number of beads required for the multiplex assay, monoplex vs multiplex, multiplex vs ELISA and interassay reproducibility of the multiplex assay were assessed. After the testing phases the blood samples were screened. This was followed by a quality control of the high positive control and a calculation of the cut-off values for sero-positivity per Ag and statistical data analysis on the screened blood samples.

3. Results

Twenty of the twenty-four Ags were selected for the assay and the six positive sera, showing the strongest signal, were pooled and used as positive control. A significant correlation was seen between the monoplex vs multiplex assay for all Ags ($p<0.001$). The ΔMFI of 1000 beads/Ag/well are almost identical to 2000 beads/Ag/well, and significantly correlated. The percent positivity obtained with the multiplex assay are lower than the results for the ELISA. A good correlation ($p<0.001$) between the assays on two different plates is seen, which showed that the results are accurate and reproducible. A comparison between the serological markers showed that a high sero-positivity and specific Ab levels were detected with LSA3-RE, CSP, Pf MSP1-19, GLURP, SALSA2 en Pf GLURP R2 Ags. PCR positive samples responded to a higher number of Ags as compared to PCR negative samples. An increase in sero-positivity for the different age-groups was observed for all Ags tested. A difference in sero-positivity was observed between the different districts.

4. Conclusion

The multiplex bead-based immunoassay was successfully implemented in the molecular malaria unit of the IPC. The method has proven to be valuable for the detection of anti-malarial Abs in human blood samples in a region with relatively low prevalence. The assay can include multiple peptides and recombinant proteins at the same time, making it more sensitive and informative as compared to regular ELISA.

Samenvatting

1. Introductie

Malaria is wereldwijd de meest voorkomende infectieziekte overgedragen door muggen en wordt veroorzaakt door een protoza van het genus *Plasmodium*. In Cambodja leven ongeveer 2.1 miljoen mensen in een risicogebied, waarvan 500.000 in de hoog-endemische jungle. Cambodja wordt gekenmerkt als een gebied met een lage malaria prevalentie en incidentie, waar het moeilijk kan zijn om de aanwezigheid van de parasiet aan te tonen. Het algemene doel van deze masterthesis is het implementeren van een bestaande multiplex assay voor de detectie van Al tegen malariaparasieten in menselijke bloedstalen. De huidige masterthesis past in het kader van een grootschalig onderzoeksproject dat gericht is op het evalueren van het effect van het massagebruik van veilige en effectieve muggenwerende middelen op transmissie van malaria, in aanvulling op het gebruik van geïmpregneerde muskietennetten. Eén van de uitkomsten in dit project is een serologische indicator.

2. Methodologie

Een assay gebasseerd op 14 *Plasmodium* specifieke peptiden LSA1-41, LSA1-J, LSA3-NR2, LSA3-RE, GLURP, GLURP-P3, SALSA1, SALSA2, CSP, SR11.1, STARP-R, Pm CSP, Pv Like CSP en PvVK210 CSP, 1 peptide (SALIV2) specifiek voor het speeksel van *An. gambiae* en 5 *Plasmodium* specifieke recombinante proteinen Pf GLURP R2, Pf MSP1-19, Pv MSP1-19, Pv DBP en Pf13 werd ontwikkeld voor het MAGPIX systeem en toegepast op de bloedstalen van 1440 individuen van de provincie Ratanakiri, Cambodja. Implementatie van de assay werd geïnitieerd met het oplossen van de verschillende antigenen (Ag), waarna een selectie van de positieve controles met ELISA werd uitgevoerd (17 willekeurig geselecteede serumstalen werden getest op 24 Ag). Vervolgens werden de beads covalent gebonden met de Ag, als eerste met 1 specifieke peptide (GLURP) voor $1*10^6$ beads en $5*10^6$ beads en vervolgens op de recombinante proteinen (op $1*10^6$). Na het testen van de koppeling werden alle Ag gekoppeld aan de beads (op $5*10^6$). Het optimaliseren van de multiplex assay werd uitgevoerd door het vergelijken van de hoeveelheden beads, van de monoplex ten opzichte van de multiplex assay, van de multiplex ten opzichte van de ELISA en door het testen van de interassay reproduceerbaarheid op de multiplex assay. Na het optimaliseren en implementeren van de multiplex assay werden alle bloed stalen gescreend. Hiervoor werd een kwaliteitscontrole van de hoog positive controle uitgevoerd en de cut-off waarde per Ag voor de sero-positiviteit bepaald om vervolgens een data analyse van de geanalyseede bloedstalen uit te voeren.

3. Resultaten

Twintig van de vierentwintig Ag werden voor deze studie geselecteerd en de zes meest positieve sera werden samengevoegd en gebruikt als positieve controle. Een significante correlatie tussen de monoplex en multiplex assay werd waargenomen voor alle Ag ($p<0.001$). De ΔMFI van de 1000 en 2000 beads/Ag/well waren bijna identiek. De percentage positiviteit (PP) bepaald met de multiplex was over het algemeen lager dat de PP in ELISA. Een goede correlatie ($p<0.001$) werd waargenomen bij het vergelijken van de multiplex assay op twee verschillende platen. Dit wijst erop dat de resultaten accuraat en reproduceerbaar zijn. Een vergelijking tussen de serologische markers laat hoge specifieke Al levels zien en een hoge sero-positiviteit werd waargenomen voor de Ag LSA3-RE, CSP, Pf MSP1-19, GLURP, SALSA2 en Pf GLURP R2. The PCR positieve stalen reageren op meer Ag PCR negatieve stalen. Met stijgende leeftijd, is er een toename in sero-positiviteit. Tenslotte is er tussen de verschillende districten, ook een verschil in sero-positiviteit.

4. Conclusie

Het implementeren van de multiplex assay is succesvol verlopen op het IPC. Deze methode is waardevol gebleken voor het testen van anti-malaria Al in menselijke bloedstalen in een gebied met een relatief lage prevalentie. Daarnaast is het mogelijk een groter bereik te creëren binnen de waarden van de positive samples in vergelijking tot een ELISA.

List of abbreviations

Abs	Antibodies
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
Ags	Antigens
AMA-1	Apical membrane antigen-1
BSA	Bovine serum albumin
CNMCP	Cambodian National Malaria Control Program
COD	Corrected optical densities
CSP	Circumsporozoite protein
DBP	Duffy binding protein
DDT	Dichlorodiphenyltrichloroethane
dH_2O	Distilled water
EDC	1-Ethyl-3-[dimethylaminopropyl]carbodiimide hydrochloride
EIR	Entomological Inoculation Rate
ELISA	Enzyme Linked ImmunoSorbent Assay
FSAT	Focused Screening and Treatment
HIV	Human immunodeficiency virus
IFA	Immunofluorescence assay
IP	Institut Pasteur
IPC	Institut Pasteur du Cambodge
IRS	Indoor residual spraying
ITM	Institute of Tropical Medicine Antwerp
ITNs	Insecticide treated nets
LED	Light-emitting diode
LSA	Liver-stage antigen
MDR	Multi-drug resistant
MFI	Median fluorescent intensity
MFIA	Multiplexed fluorescent immunoassay
MOH	Ministry of Health
MRDDs	Malaria Rapid Diagnostic Devices
MSP	Merozoite surface protein
NH_4OH	Ammonium hydroxide
OD	Optical densities
PBS-CR	Phosphate buffered saline-Charles River
PCR	Polymerase chain reaction
PE-labeled det. Ab	Phycoerytrin-labeled detection antibody
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein
PP	Percent positivity
QBC	Quantitative Buffy Coat
RBCs	Red blood cells
RBM	Roll Back Malaria
RDTs	Rapid diagnostic tests
SALSA	Sporozoite- and liver-stage antigen
STARP	Sporozoite threonine- and asparagines rich protein
Sulfo-NHS	N-hydroxysulfosuccinimide
TB	Tuberculosis
TNF	Tumor necrosis factor
TRAP	Thrombospondin-related anonymous protein
WHO	World Health Organization

1 Introduction

1.1 Malaria in general

Malaria, the most common mosquito-borne infection in the animal world, is caused by a protozoan parasite of the genus *Plasmodium*. Human malaria is caused by one of four *Plasmodium* species (*Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Plasmodium falciparum*), although a fifth species common in monkeys (zoonosis) can also infect humans (*Plasmodium knowlesi*) [1, 2]. Malaria caused by *P. falciparum* is the most severe form of malaria that can be fatal and predominates especially in Africa. *P. vivax* is rarely fatal, and occurs mostly in areas outside of Africa. The other two species, *P. ovale* and *P. malariae* are less frequently found. In Southeast Asia the species *P. falciparum* and *P. vivax* predominate [1, 3, 4].

Malaria parasites are transmitted through the bite (from sunset to sunrise) of an infectious female mosquito. Of more than 3200 existing mosquito species *(Culicidae),* only 70 to 80 species all belonging to the genus *Anopheles* [1] are able to act as a vector of human malaria. The lifecycle of these mosquitoes, requiring a blood-meal for female mosquitoes before laying eggs, is of great importance for the transmission of the *Plasmodium* parasite [1].

The occurrence of malaria varies widely, not only from geographical region to region. Moreover, great heterogeneity in malaria transmission may occur between villages and even within one village. The transmission of the parasite depends on the mosquitoes age, the ambient temperature, the population density of both mosquito-vectors and humans, the mosquito's human-biting habit, the immune-system of the host, as well as the use of control measures such as vector control and treatment of diagnosed cases [2]. Depending on the intensity of the transmission, malaria can be stable or unstable. Stable malaria means that the malaria infection rate does not fluctuate according to seasons despite fluctuation in transmission and malaria morbidity. Malaria transmission is intense throughout the year and is steady from year to year. In areas with stable malaria, the clinical disease is mainly characterized by affecting young children (under the age of five), pregnant woman, and less adults due to acquired protective immunity. In areas with unstable malaria the transmission tends to be low and varies significantly from year to year. In these areas epidemics can occur affecting all age groups as acquired immunity is very low or almost absent [2].

The reduction of mortality and morbidity can be realized, by vector control through indoor residual spraying (IRS) and distributing insecticide treated nets (ITNs), but unfortunately, many people still do not have access to ITNs [3, 5]. In addition, the most susceptible populations, especially young children and pregnant woman, can be protected by intermittent preventive treatments. An important backbone of control programmes is also the case management relying on the diagnosis for every suspected case through microscopy or rapid diagnostic tests (RDTs) and subsequent treatment. Still many people are incapable to access life-saving malaria treatment within 24 hours after clinical symptoms appear [3].

1.2 Global problem of Malaria, and specific situation in Southeast Asia

Malaria is one of the major worldwide causes of death from infectious disease, together with Human immunodeficiency virus (HIV) and Tuberculosis (TB). Malaria is a public health problem for 3.3 billion people of the world population in 107 malaria-endemic countries. Of this total, 1.2 billion are at high risk mainly in the African (47%) and South-East Asian (37%) regions. In 2010, the number of malaria cases was estimated at about 216 million, and still about 655.000 people of all ages die of malaria. About 86% of all deaths were in children under the age of 5 years. Between 2000 and 2010 the global malaria incidence dropped with about 17% and the number of deaths with about 26% [3].

Despite this large drop in morbidity and mortality, in Southeast Asia still 70% of the 1.8 billion people are at risk for malaria, of which 25% are at high risk. In 2010, 4.3 million cases of malaria and 2426 malaria related deaths have been reported in this region of which 2.4 million cases were parasitologically confirmed, mainly in India (66%), Myanmar (18%) and Indonesia (10%) [3].

In Southeast Asia national malaria control campaigns have been launched, under direct control and encouragement of the World Health Organization (WHO) between the late '40 and '50. These campaigns were based on indoor spraying with dichlorodiphenyltrichloroethane (DDT). This resulted in a spectacular decrease in malaria incidence and malaria-related mortality, especially in India, where simultaneously use of health care delivery systems providing treatment with anti-malarial drugs, in particular chloroquine, were improved. Despite this success, elimination of malaria was only achieved in a few regions in Asia and was not strong enough in order to achieve a global extermination [6]. In 2007, the Bill & Melinda Gates Foundation re-introduced the concept of eradication of malaria with the support of the WHO, Roll Back Malaria (RBM) partnership and many other organizations. In order to achieve this goal the countries have to undergo different well-defined control phases, such as pre-elimination, elimination and prevention of re-introduction [7]. However, it is clear that currently we do not have all tools to completely interrupt the transmission cycle [7]. Therefore, many endemic countries set up strategies to eliminate malaria, for example Cambodia. Cambodia launched a national strategy plan in 2011, under direct control of the first minister Hun Sen, for malaria elimination in 2025 [8]. The current research project is located in Cambodia, in the province of Ratanakiri.

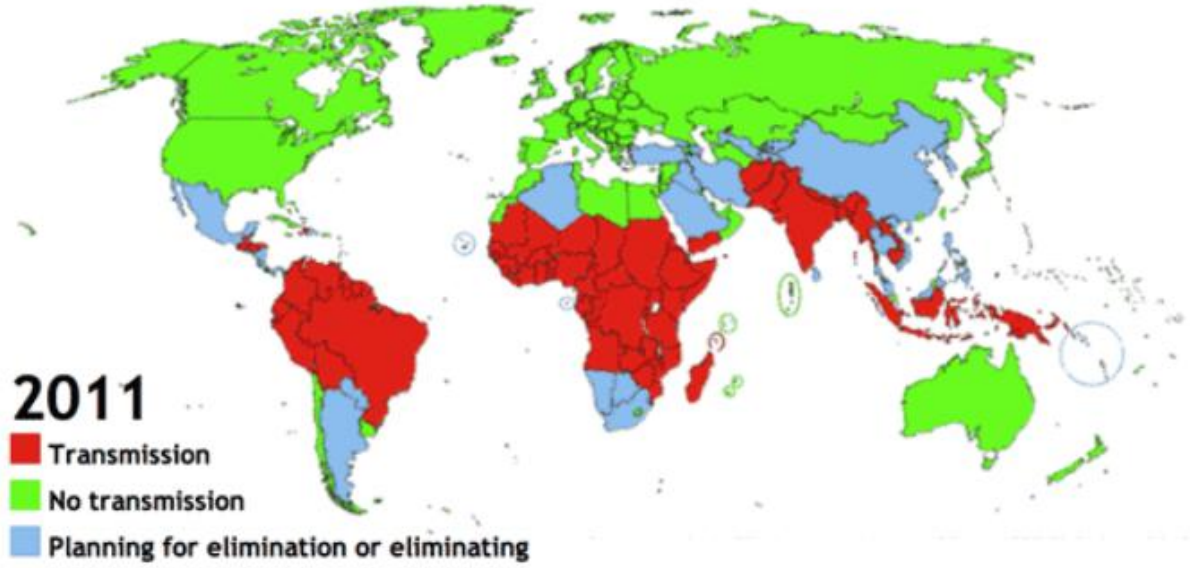


Figure 1: malaria transmission countries [9].

1.2.1 Malaria in Cambodia

Cambodia is knew for its history characterized by civil wars and genocide by the Khmer Rouge in the period between 1975 and 1979 [10]. A few years before these atrocities in 1951 the Ministry of Health (MoH) in Cambodia started Malaria control operations with the support of the WHO. They created a program for malaria treatment and vector control. This was a successful program that ended when the Khmer Rouge invaded Cambodia in 1971. The regimen repressed the population and deported most of them to high endemic regions [8]. Particularly health workers and teachers were under attack, of which about 2 million died of violence, malnutrition and disease, and only a few survived the Khmer Rouge. From the 1980s, Cambodia began to rebuild its health system focusing on a growing number of professional health workers [10].

Nowadays, malaria is still a public health problem for 53% of the people living in Cambodia. About 2.1 million people in Cambodia are at risk, of which about 500.000 people live in the high endemic forested areas [1, 5]. Due to a successful national anti-malaria program the mortality rate decreased between 1998 and 2011 from 629 to 67 deaths per 100.000 people, while the Cambodia's Millennium Development Goal of 2015 was set at 78 deaths per 100.000 people. During the last years the programme was very performing: between 2010 and 2011, the number of malaria related deaths dropped from 151 to 94, which is a decrease of 38% [8, 11]. On the other hand, there was a

slight increase of 7% in malaria cases that has been reported between 2010 (58.621 people) and 2011 (62.770). In this thesis I will focus on the province of Ratanakiri were the sera samples have been collected for the current research project. In the province of Ratanakiri the cases of deaths dropped with 71% in one year (from twenty-two persons in 2010 to five persons in 2011 and none in 2012) [11]. In the past *P. falciparum* malaria cases were more frequent than *P. vivax* cases, but with the implement control measures the reverse situation occurs.

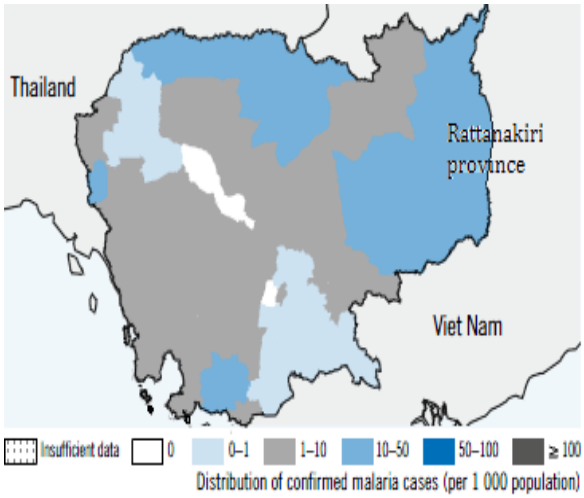


Figure 3: malaria in Cambodia [3].

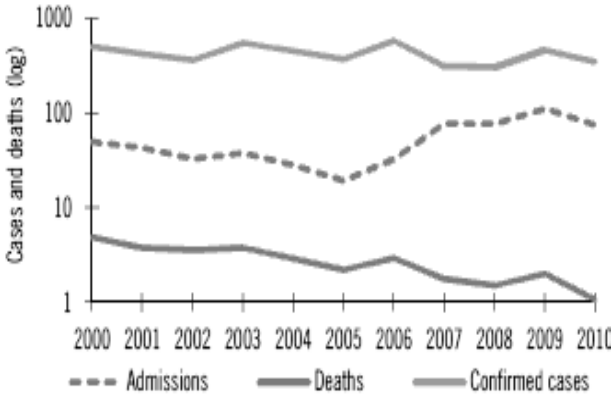


Figure 2: Confirmed cases in Cambodia (from 2000 to 2010 [3].

Different vector species can be identified in Cambodia. These are the "major primary vector", and "secondary vector". There are two major primary vector species, *Anopheles dirus,* and *Anopheles minimus* that cause malaria transmission all year round [12]. The importance of the secondary vector species still has to be confirmed [1, 5].

1.3 Life-cycle and pathogenesis

Malaria cannot be transmitted directly between humans, apart from exceptional cases of medical blood transfusion and perinatal mother-to-child transmission. Passage of the parasite through an anopheline mosquito is required for the transmission. The *Plasmodium* life-cycle (Figure 4) starts with the asexual phase in the vertebrate host. The sexual phase is completed in the vector [1]. To complete these cycles, three components are needed; namely the vertebrate host (human), the vector (in Cambodia *An. dirus* and *An. minimus* are the main vectors) and the parasite [1, 2].

1.3.1 Schizogonic cycle

The basic elements are the same for all *Plasmodium* species. Transmission starts with the bite of a female *Anopheles* mosquito inoculating plasmodial sporozoites into the host. After a few minutes the sporozoites migrate to the liver and invade the hepatocytes. Within the hepatocytes the parasites transform into tissue schizonts (=pre-erythrocytic or exo-erythrocytic stage, Figure 4) [1]. Each schizont produces several thousand merozoites, which are released after 8-10 days back into the bloodstream and enter the erythrocytes [1]. Some merozoites released by the hepatic schizonts are destroyed by the immune system in the plasma. Within the red blood cells the parasites are transported through the circulation and undergo transformation into trophozoites. These trophozoites develop into mature erythrocytic schizonts [13]. The schizonts produce about 16 to 32 merozoites [1]. After 48 hours *(P. falciparum, P. vivax and P. ovale)* or 72 hours *(P. malariae)* the erythrocytes rupture and release more merozoites into the plasma, where they rapidly invade new healthy erythrocytes, thereby perpetuating and promoting the schizogonic cycle. This rupture of erythrocytes causes the typical recurrent fevers observed in malaria [1, 14].

Other trophozoites develop after 9 to 10 days into female and male gametocytes. Gametocytes do not develop further in humans and once ingested by a mosquito they undergo the sexual phase [1].

In contrast to *P. falciparum* and *P. malariae*, sporozoites from *P. vivax* and *P. ovale* can transform into hypnozoites, which stay in the liver in a sleeping form. After 1 to 12 months these hypnozoites might relapse after apparent cure [1].

1.3.2 Sporogonic cycle

The sporogonic cycle (Figure 4) starts when an *Anopheles* mosquito ingests female and male gametocytes during a blood meal. In the stomach, the intracellular gametocytes differentiate into female and male extracellular gametes. The male gametocytes exflagellate into eight sperm-like flagellated microgametes, each of which when conjugated with a female macrogamete generates a zygote. This zygote, or motile ookinete, penetrates the peritrophic membrane and settles in the outer gut wall to form a non motile oocyst which immediately starts dividing. Each oocyst can produce up to 8.000 sporozoites to release into the body cavity. The time required for the oocyst to burst depends on the *Plasmodium* species and the external conditions (e.g. temperature) and varies from 7 to 20 days [1]. Then, the sporozoites migrate to the salivary glands. From the moment they reach the salivary glands, the mosquito becomes infectious and is able to actively inject the sporozoites into the vertebrate host to start the asexual cycle again [1].

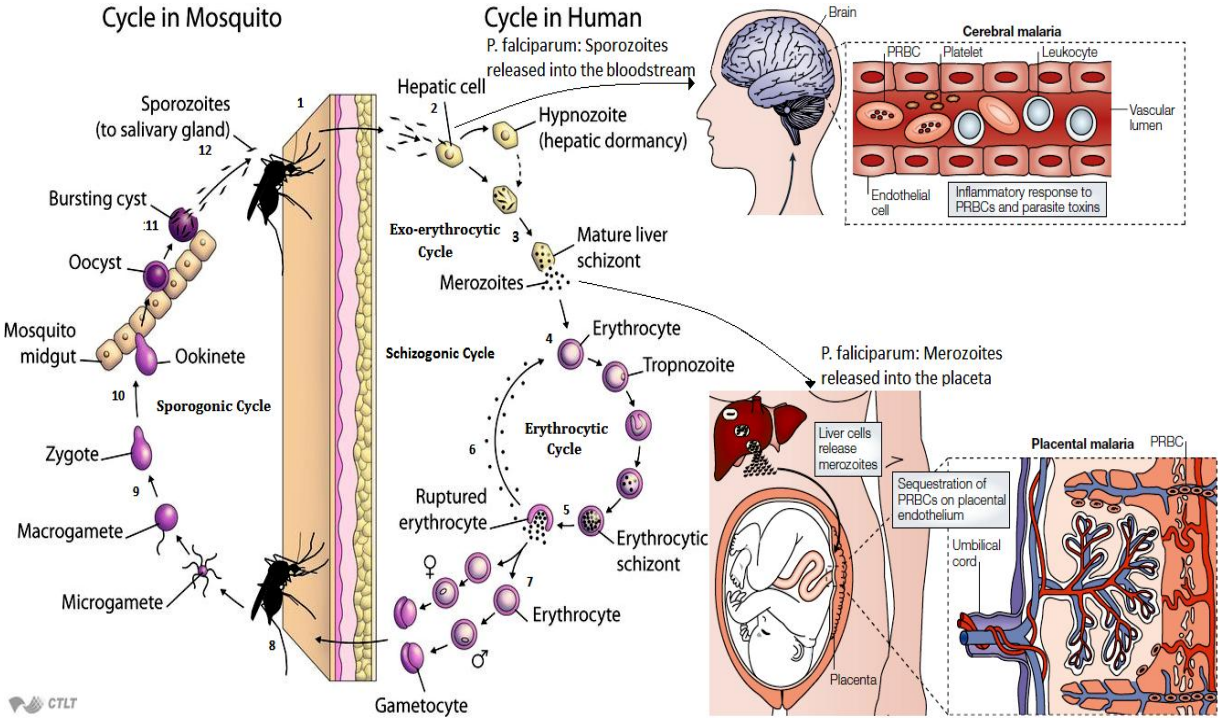


Figure 4: The lifecycle of the *plasmodium* parasite and *P. falciparum* [13, 15].

1. Mosquito takes a blood meal and injects sporozoites into the human's bloodstream. Start of the schizogonic cycle; 2.The sporozoites infect the hepatocytes. Sporozoites replicate and transform into schizonts; 3.After 8-10 days the schizonts rupture and release merozoites into the bloodstream; 4. The merozoites enter the erythrocytes and start the erythrocytic cycle and transform into ring-stage trophozoites and transfer into erythrocytic schizonts. The schizonts produce about 16-32 merozoites; 5.After 48-72 hours the schizont ruptures and releases merozoites; 6.Some merozoites transform back into trophozoites; 7.Other merozoites invade new healthy erythrocytes and transform into male and female gametocytes; 8.Mosquito takes a blood meal and ingests the gametocytes → start of the sporogonic cycle; 9.Gametocytes enter the midgut → male gametocytes penetrate the female gametocytes and produce zygotes; 10.Zygote transforms into a slow motile ookinete. Ookinete penetrates the midgut and develops into an oocyst; 11.Oocyst produces ± 8.000 sporozoites → oocyst bursts; 12.Sporozoites migrate to the salivary glands and settle in the salivary duct of the mosquito.

1.4 Immunological response in the human host

Inoculation of parasite molecules in the blood stream of the human host triggers an immune response, regulated by the innate (first line native defense system) and the adaptive immune system

(humoral and cell-mediated immune responses) [16–18]. This process involves protection against the extracellular (sporozoites and merozoites), and the intracellular liver and blood-stage [13, 15]. There are different levels of immunity against malaria parasites. When a non-immune person gets infected for the first time, he/she will develop acute clinical symptoms even at low levels of parasitemia, which can evolve into a severe form and even death [6, 19]. Then, when a person is infected several times, anti-disease immunity develops, which is a defense mechanism against clinical symptoms even at high levels of parasitemia and decreases the risk of severe malaria [6]. Furthermore, repeated infections by full exposure leads to anti-parasite immunity. This is a protection against high levels of parasitemia and results in low levels of parasitemia without clinical symptoms. When immunity is acquired, it will be temporary [6, 19].

When a child is born from a malaria immune mother, it has passive immunity against malaria during the first 6 month of his/her life. This is due to the transfer of maternal antibodies from mother to child, and is followed by an increased susceptibility for malaria for one or two years before acquiring active immunity. In general, obtaining active natural immunity against malaria takes time and requires repeated parasite exposure [13, 15]. Acquired immunity decreases the risk of both severe and mild malaria but does not suppress the occurrence of a malaria infection. The defence mechanisms necessary to reduce the parasitemia is based on the innate protection (e.g. G-6-PD deficiency, drepanocytosis) and the acquired immunity based on the humoral and cell-mediated response [20]. In the following I will focus on the humoral immunity as in this master thesis, the aim is to detect anti-malarial antibodies.

1.5 Antibody-related protection

As mentioned before most of the people who live in highly endemic areas have developed certain immunity to malaria in which antibodies (Abs) play a major role [21]. Anti-malarial antibodies may be protective by reducing parasitemia and disease through a variety of mechanisms [17]. The expression of parasite antigens (Ag) on the surface of *P. falciparum* infected erythrocytes is mainly important for generating this response (Figure 5). The parasite can avoid the immune system because of its intracellular development and a large variability of parasite Ags. These Ags have v*ar*-genes including erythrocyte membrane protein 1 (PfEMP-1) and *rif*-genes [17].

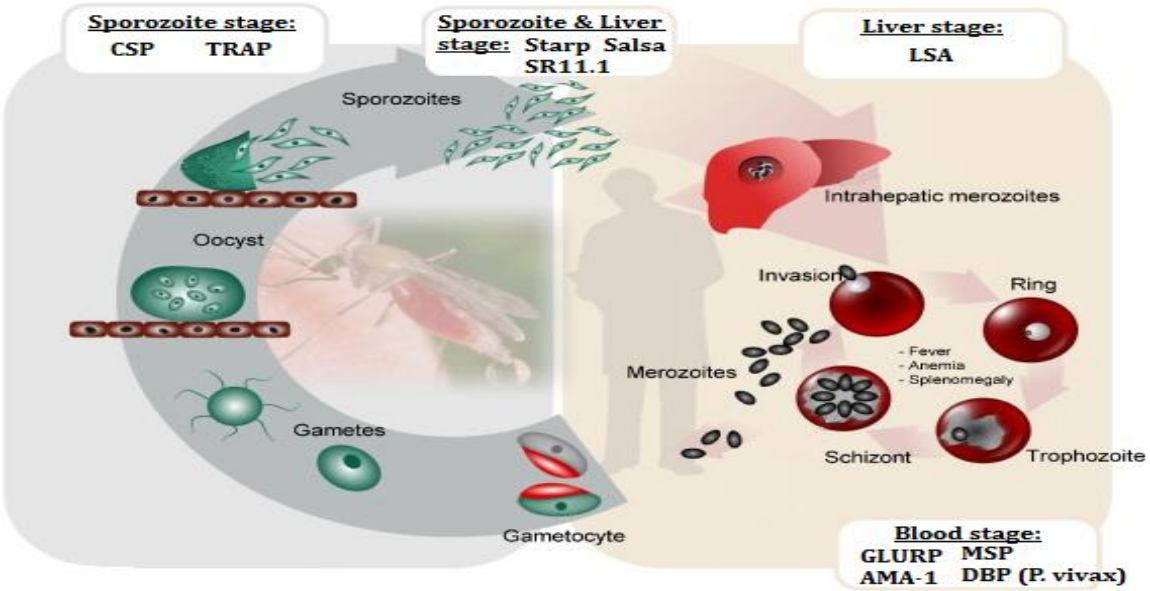


Figure 5: Plasmodium life cycle and the parasite antigens [22].
This figure shows at which stage within the life-cycle the Ags are expressed by the parasites.

Anti-sporozoite antibodies, targeting for example circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP), are directed against proteins on the sporozoite

surface [23, 24]. It is suggested that these Abs are involved in protective immunity by inhibiting the sporozoite entry, but they do not totally stop the sporozoite entry [23]. Sporozoite threonine- and asparagines rich protein (STARP), sporozoite- and liver-stage antigen (SALSA) and sub region antigen SR11.1 (represents to a unique subregion of the megaprotein Pf11.1) are pre-erythrocytic antigens that both express at the sporozoite and *P. falciparum* liver stages [25–27]. A high Ab response against these Ags is frequently found in persons living in malaria-endemic areas and induced a decrease of the sporozoite invasion into the liver cells [25, 26]. In contrast to other pre-erythrocytic Ags, liver-stage antigens (LSA) are one of the few antigens only expressed in the liver-cells [24, 28]. The way of expression to the immune system differs from anti-sporozoite antigens. LSA expression appears quickly after sporozoite invasion of the liver cells and enhance during the liver cycle. LSA is with the developing parasite localized in the parasitophorous vacuole as a separated flocculent material and play a crucial role during the schizogonic cycle by protecting the merozoite surface [24, 28].
Anti-merozoite antibodies, targeting for example merozoite surface protein (MSP) and apical membrane antigen (AMA-1), contribute to inhibiting a merozoite invasion of erythrocytes, intra-erythrocytic growth and they can improve removal of infected erythrocytes from the circulation by attaching to the erythrocytic surface [17, 29]. Duffy binding protein (DBP) is an Ag that is only expressed on the merozoite surface in *P. vivax* infected persons. The anti-DBP-antibodies play a crucial role in the erythrocyte invasion by blocking the DBP binding to the Duffy antigen-receptor [30]. Opsonization of infected erythrocytes raises their susceptibility to phagocytosis, cytotoxity and inhibition by parasites effector cells of the immune system [17]. Interaction between opsonized erythrocytes with the parasite effector cells, leads to a production of factors, such as tumor necrosis factor (TNF) [16, 17].
While the specificity of protective Abs is still unclear, it is known that several contribute to malaria immunity [21]. This master thesis will focus on the detection of Abs against Ags that are expressed by different stages of the *Plasmodium* parasite.

1.6 Detection methods

An accurate diagnosis of malaria on technical grounds is very important in a variety of settings, for clinical, epidemiological and research purposes. A clinical suspicion of malaria should be treated accordingly because the symptoms of complicated malaria can develop suddenly, which may result to death. For this reason, laboratory tests are required to be performed in order to confirm the presence of the malaria parasite in the patient, if possible [31]. To diagnose malaria, a direct method is used. The direct diagnosis is based on the detection of the living parasite, its Ag or its DNA in the blood. The detection of Abs (indirect diagnosis) is used to confirm the parasite exposure [1]. The main used direct methods for diagnosis are: conventional microscopic diagnosis by staining thin and thick blood smears, the Quantitative Buffy Coat (QBC) method, the Rapid Diagnostic Test (RDT), molecular diagnostic methods, such as polymerase chain reaction (PCR). For indirect diagnosis, the Enzyme Linked ImmunoSorbent Assay (ELISA), is usually used as immunological test [31].

1.6.1 Microscopic diagnosis

Conventional microscopic diagnosis by staining thin and thick blood smears is the oldest routinely and still used method to estimate the parasite load [1, 31]. A minimum of equipment is required: a staining solution and a microscope with an immersion lens. However, this technique requires intensive training and must be carried out with precision [1]. In this technique malaria is microscopically visually diagnosed by staining thick and thin blood films on a glass slide. Two drops of blood are taken from a patient's finger and put on a glass slide [31].
Thin blood smear procedure is less sensitive but easier to interpret. This method allows the performer to detect a limit of 50-100 parasites/µl blood, in which detection of various *Plasmodium* species is feasible when appropriately trained [1, 32]. Thick blood smear analysis detects up to 4-20 parasites/µl blood but demands high skills [1, 32, 33]. Screening the slide requires a shorter time, but

it is very difficult to distinguish *Plasmodium* species in mixed infections, especially between species at trophozoite stage [1].

1.6.2 Quantitative Buffy Coat method

The QBC method is a method based on the microscopic diagnosis and was designed to improve the detection threshold and simplify the malaria diagnosis. This method involves staining isolated parasitized red blood cells (RBCs) in a capillary tube with a fluorescent label, acridine orange. Although, this technique is easy to learn, it requires specialized instrumentation, high costs and is poor at determining varies species of the parasite. For this reason this technique is no longer in use [1, 31].

1.6.3 Rapid Diagnostic test (RDT)

The RDT also known as Dipstick or Malaria Rapid Diagnostic Devices (MRDDs), is a sensitive test developed as an alternative to microscopy. RDT is a lateral flow immuno-chromatographic Ag-detection test that analyzes the presence of Ag in the human host by using polyclonal or monoclonal dye-labeled Abs [1, 34]. Current RDTs have a detection limit of about 100-200 parasites/µl blood *(P. faciparum)* and > 500 parasites/µl blood *(P. vivax)* which is generally above the threshold of microscopy that detects about 4-100 parasites/µl blood [33, 34]. Before testing the patient's blood, it needs to be mixed with a lysing agent that ruptures the erythrocyte so that more parasite Ags are released. Then, this Ag binds to a dye-labeled Ab and passes a nitro-cellulose strip through capillary action. This crosses a test and control line coated with a target Ab that creates a visual line when the parasite-dye-labeled Ab attach to the target Ab [34].

This test plays a major role as malaria point-of-care. RDT is a frequently used test in Cambodia to ensure improved patient management in malaria endemic areas, where no microscopes are available [1, 34], and contributes greatly to early diagnosis and subsequent treatment as it can be performed in the villages itself.

1.6.4 Molecular diagnostic methods

Techniques based on PCR are more specific and sensitive to detect malaria cases with low parasitemia or mixed infection than the above mentioned techniques. PCR can detect 1-5 parasites/µl blood (≤ 0.0001% of infected RBCs) in comparison with approximately 4-100 parasites/µl blood detected by the microscope or RDT [31, 33]. In addition, PCR can be useful in the detection of drug-resistant parasites (when a molecular marker is available), mixed infections and can be automated to process a large number of samples [31]. So, it seems to be the best method for malaria diagnosis so far, but despite this there are disadvantages, namely the complexity, the high costs of equipment and consumables and the special training for staff which would be required. As a result, PCR is still not routinely performed in developing countries [31]. However, in 2010 an epidemiological tool named "Focused Screening and Treatment" (FSAT) was introduced in Cambodia by the Cambodian government's National Centre for Parasitology. This is based on active molecular detection of symptomatic and asymptomatic carriers with subsequent treatment [5, 35] and is being carried out in the so-called 'elimination' area in the West of Cambodia with the aim to contain the potential spread of multi-drug resistant (MDR) *P. falciparum* malaria from Pailin to other regions.

1.6.5 Serology

Serological tests are based on the detection of Abs against Ags of malaria parasites. Serology is not used for diagnosis, but to see if people have been in contact with the parasites. While PCR prevalence data will provide a snapshot of the endemicity at a certain moment, sero-prevalence will provide a picture of the endemicity over a larger period. The Ab half-life is essential for a good interpretation of the results. The most commonly sero-epidemiological techniques used are the indirect immunofluorescence assay (IFA) and the Enzyme Linked ImmunoSorbent Assay (ELISA) (Figure 6) [27, 31]. IFA has been a reliable serological test for malaria, and is mainly used in

epidemiological analysis to detect *P. falciparum* or *P. vivax* specific Abs in blood. Unfortunately, even though IFA is simple, specific and sensitive it is also time consuming and interpretation of the results is subjective [27, 31]. Despite the fact that IFA is considered a standard diagnostic Ab test, it is preferred to use a simpler micro-ELISA plate for mass screening of blood samples [36].
This test involves indirect ELISA and Sandwich ELISA. In malaria serology mainly indirect ELISAs have been used. This starts with coating the well with an Ag. Blocking buffer is used to block the other protein-binding sites. Blood serum is then added for the primary Ab that would be present in the serum to bind, followed by an added detection Ab that binds to the primary Ab which is conjugated with either a colorant or an enzyme for detection of the reaction (Figure 6) [37]. This test can also be done starting from blood that is collected on filter paper [38].

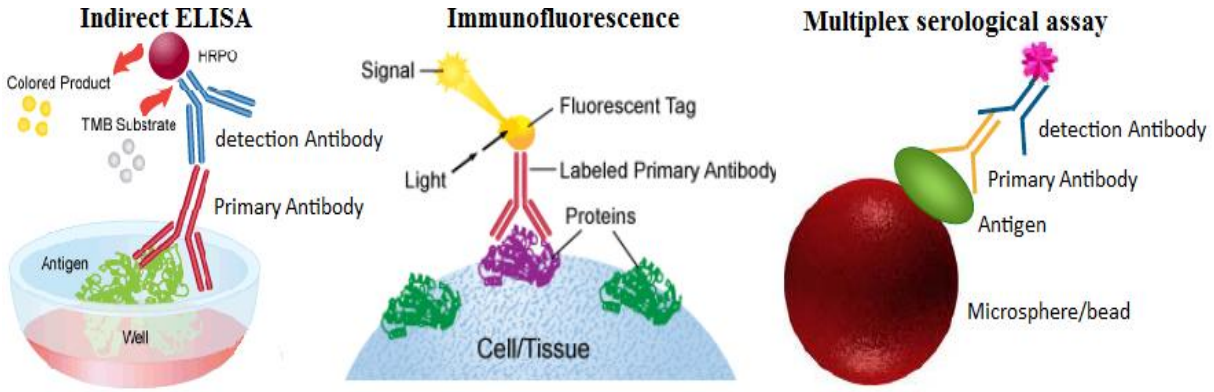


Figure 6: Differences between serological techniques [37, 39].

To improve these tests, new techniques, such as multiplex bead assays have been developed by the Luminex Corporation. The read-out systems are the Luminex 200, FLEXMAP 3D and the MAGPIX systems. With this technique we can detect multiple Ags in large populations so that it can be used as high throughput screening. The Luminex 200 and FLEXMAP 3D combines flow cytometry, microspheres (Microplex (polystyrene) or Magplex (polystyrene paramagnetic) beads), lasers and a detection system that is capable of measuring more analytes at the same time [40, 41]. For this research project based on implementing an existing assay for the detection of Abs against malaria parasites in blood samples, the Luminex-MAGPIX-system is used. The MAGPIX is a smaller compact device than the other Luminex-systems. It is not based on flow cytometric for detection, but uses light-emitting diode (LED) illuminated flow for excitation and a CCD camera for detection [42]. The MAGPIX is exclusively designed for the use of MagPlex polystyrene paramagnetic microspheres (beads) as substrate. The principle of these techniques is based on the ELISA, but instead of coating a well with an Ag, different magnetic beads will be coated with a specific Ag so when multiplexed in one assay, the different beads can capture and quantify various Abs in one blood sample. Magnetic beads will be kept in the wells by using a magnetic plate during the protocol [42, 43].
These techniques turned out to be quicker, more precise, sensitive and reproducible than the traditional ELISA and IFA procedures [27]. The Luminex 200 is capable of quantifying up to 100 beads, the FLEXMAP 3D up to 500 beads and the MAGPIX up to 50 different bead species at the same time in a sample volume. Furthermore, the time and costs are lower than the ELISA (Figure 6) [41, 42, 44]. In the current research project, in which 20 malaria specific Abs were detected at the same time in one sample, the ELISA would be very time consuming, because with the ELISA each specific Ab is analyzed separately [45].

1.6.5.1 ELISA performed in South-East Asia

For estimating the malaria transmission or parasite prevalence the entomological inoculation rate (EIR) is used as an important indicator. The EIR per night is calculated as the number of mosquito bites per man per night multiplied by the proportion of those bites positive for sporozoites [46]. This method is used as a standard for measuring the malaria transmission rate. However, with the

decreasing transmission it is difficult to find a positive mosquito which makes this method very labour intensive. Nowadays, serology based on ELISA is widely used to estimate the malaria transmission [46].

The ELISA is also used to screen serum samples from malaria positive patients for the presence of Abs to the different Ags. Table 1 shows several serological studies in Southeast Asia that shows that in Thailand, Malaysia and Myanmar is a large variation in sero-prevalence, depending on which Ag is tested. In Cambodia about 2-5% is tested positive for anti-*P. falciparum* antibodies and about 6-8% is tested positive for anti-*P.vivax* antibodies.

Country (reference)	Year of Study	Antigens	Total number of ELISA	Number of tested positive	Age groups	Sero-prevalence (%)	
Thailand	1986	*P. falciparum* antibodies	-	-	-	Village 6: 48.9% Village 7: 28.8% Village 11: 84.6%	[47]
Malaysia	1988	PvCSP + PfCSP	94	44		46.8%	[48]
Thailand	1989	Gambian strain of *P. falciparum*	418 288 3334 574 860 122 319 89	-	0-15 male 0-15 female 16-30 male 16-30 female 31-45 male 31-45 female >45 male >45 female	7% 5% 56% 10% 14% 2% 5% 1%	[49]
Malaysia	1990	*P. falciparum* antibodies	-	-	0-4 30-39 >40	*P. falciparum* 32-47% *P. vivax* 16-30% *P. malariae* 7-19%. *P. falciparum* 5-11% *P. vivax* 0-7% *P. malariae* 0-12%. *P. falciparum* 8-19% *P. vivax* 4-14% *P. malariae* 1-5%.	[50]
Thailand	1990	PvCSP	804	217	-	26.99%	[51]
Malaysia	1991	CSP	268 268 268	14 30 6	-	*P. falciparum* 5.2% *P. vivax 11.2%* *P. malariae 2.2%*	[52]
Thailand	1992	Pf115/RESA *P. vivax*	421 421	63 (39) 63 (17)		61.9% 27%	[53]
Thailand	1994	Pf155/RESA	20 22 39 37	10 19 34 33	0-15 16-30 31-45 >45	50% 86% 87% 89%	[54]
Vietnam	2001	Pf Anti-MSP4 (EcMSP4-His) Pf Anti-MSP4 (ScMSP4-His) Pf Recombinant MSP1-19	342 342 174	-	-	82.2% 93.9% 96.5%	[55]
Cambodia (August)	2005	Pf GLURP Pv MSP1-19	804 804	37 64	-	4.6% 7.96%	[38]
Cambodia (November)	2005	Pf GLURP Pv MSP1-19	804 804	16 48	-	1.99% 5.97%	[38]
Thailand	2007	PvMSP1	37 84 67 12	16 43 40 11	13-17 18-25 26-40 >40	43% 51% 60% 11%	[56]
Myanmar	2011	Lsa-1 Lsa-3 PvVK210-CSP PvVK247-CSP	112 112 112 112	82 33 36 18	-	73.2% 29.5% 32.1% 16.1%	[57]

Table 1: ELISA studies in Southeast Asia [38, 51, 57].

1.6.5.2 Studies performing multiplex assays

For testing more Ags at the same time a multiplex assay is used. This multiplex assay has never been used in Southeast Asia, but it has been implemented in other parts of the world. Table 2 shows several multiplex assay studies performed in different areas in Africa. This shows that multiple Ags can be tested at the same time instead of one. The results show that Lsa1-41 (87%), Glurp (100%), STARP-R (80%) and CSP (96%) give the highest results in positivity in sero-prevalence. Medium results are found in LSA3-RE (52.5%), LSA1-J (58%). SALSA1 (53%), SALSA2 (59%) and SR11.1 (64%). The other Ags show lower results between 0-50 percent. However, as human and parasite populations between regions or continent might be different, it is important that the antigenicity of the Ags is tested in the population of interest.

Country (reference)	Year of study	Peptides/proteins	Total # of samples tested	Number of tested positive	Sero-prevalence (%)	
Republic of Djibouti	2002	Lsa1-41, Lsa1-J, Lsa3-NR2, Glurp, GlurpP3, Salsa1, Salsa2, Trap1, Starp-R, CSP and SR11.1. PfMSP1-19, AMA1, PvMSP1-19	1910 1910	602 334	*P. falciparum* 31.5%* *P. vivax 17*.5%**	[58]
Senegal (travelers)	1995	Lsa1-41	124	7	5.6%	[27]
		Lsa1-J	124	4	3.2%	
		Lsa3-NR2	124	1	0.8%	
		Lsa3RE	124	0	0.0%	
		Glurp	124	5	4.0%	
		GlurpP3	124	1	0.8%	
		Salsa1	124	0	0.0%	
		Salsa2	124	2	1.6%	
		Trap1	124	0	0.0%	
		Trap2	124	0	0.0%	
		Starp-R	124	1	0.8%	
		CSP	124	7	5.6%	
		SR11.1.	124	1	0.8%	
		Saliv 1	124	0	0.0%	
		Saliv 2	124	1	0.8%	
Senegal (Diama village)		Lsa1-41	38	19	50%	[27]
		Lsa1-J	38	4	11%	
		Lsa3-NR2	38	3	8%	
		Lsa3-RE	38	0	0%	
		Glurp	38	25	66%	
		GlurpP3	38	7	18%	
		Salsa1	38	7	18%	
		Salsa2	38	16	42%	
		Trap1	38	0	0%	
		Trap2	38	0	0%	
		Starp-R	38	3	8%	
		CSP	38	10	26%	
		SR11.1.	38	9	24%	
		Saliv 1	38	3	8%	
		Saliv 2	38	0	0%	

Senegal (Ndiop village)		Lsa1-41	40	33	83%	[27]
		Lsa1-J	40	15	38%	
		Lsa3-NR2	40	7	21%	
		Lsa3-RE	40	0	9%	
		Glurp	40	39	80%	
		GlurpP3	40	21	34%	
		Salsa1	40	23	34%	
		Salsa2	40	23	59%	
		Trap1	40	3	9%	
		Trap2	40	0	9%	
		Starp-R	40	15	21%	
		CSP	40	32	43%	
		SR11.1.	40	12	40%	
		Saliv 1	40	2	21%	
		Saliv 2	40	0	9%	
Senegal (Dielmo village)		Lsa1-41	45	39	87%	[27]
		Lsa1-J	45	26	58%	
		Lsa3-NR2	45	15	33%	
		Lsa3-RE	45	3	7%	
		Glurp	45	45	100%	
		GlurpP3	45	15	33%	
		Salsa1	45	24	53%	
		Salsa2	45	43	96%	
		Trap1	45	2	4%	
		Trap2	45	1	2%	
		Starp-R	45	36	80%	
		CSP	45	43	96%	
		SR11.1.	45	29	64%	
		Saliv 1	45	5	11%	
		Saliv 2	45	0	0%	
Northern Senegal (June)	2004	Lsa1-41	59	7	11.9%	[59]
		Lsa1-J	59	14	23.7%	
		Lsa3-NR2	59	31	52.5%	
		Glurp	59	10	16.9%	
		Salsa1	59	9	15.2%	
Northern Senegal (Sept)	2004	Lsa1-41	62	12	19.3%	[59]
		Lsa1-J	62	10	16.1%	
		Lsa3-NR2	62	25	40.3%	
		Glurp	62	11	17.7%	
		Salsa1	62	16	25.8%	
Northern Senegal (Dec)	2004	Lsa1-41	65	17	26.1%	[59]
		Lsa1-J	65	12	18.5%	
		Lsa3-NR2	65	32	49.2%	
		Glurp	65	13	20.0%	
		Salsa1	65	16	14.6%	

Table 2: several multiplex assays [27, 58, 59].

***Based on positivity to at least two different peptides or recombinant proteins of the 13 *P. falciparum* Ags used in this study.**

****Based on positivity to PvMSP1-19 or PvMSP1-42**

2 Project and objective

The current master thesis fits within the framework of a large-scale research project entitled 'Repellents as added control measure to long lasting insecticidal nets to target residual transmission in Southeast Asia: a step forwards to malaria elimination'. This project is managed by the 'Institute of Tropical Medicine Antwerp' (ITM) in collaboration with the Cambodian National Malaria Control Program (CNMCP) and 'Institut Pasteur du Cambodge' (IPC). The purpose of the project is to acquire knowledge about the effect of mass use of safe and effective mosquito repellents on the malaria transmission, in addition to the use of impregnated mosquito nets. If this strategy is effective, it could be essential for achieving elimination of malaria in Cambodia by 2025.

One of the outcomes of the project is based on serological markers. In areas with a low prevalence and incidence of malaria, such as Cambodia, detection of parasitological indicators by microscopy and to lesser extent by PCR can be insensitive. Serological tests are more sensitive in detection of malaria exposure and can be used as an approach to measure the transmission intensity [38]. Therefore it is important to determine Ab titers within populations in low endemic malaria areas. A classically used method is the ELISA. In this technique, each Ag is tested separately, which makes ELISA labor-intensive and time-consuming. However, often, only small amounts of blood are taken, for example through finger-prick blood samples from adults. Therefore, an assay that measures Abs to multiple Ags in a few micro liter of blood is needed. This kind of immunoassay has been developed using the Luminex technology [21]. In the repellent project such assay will be used to measure changes in the force of infection due to different malaria control measures (repellent versus no repellent). Implementation of this assay will complement the expertise that is currently available at the IPC, as this assay could be used in future research projects and will improve the understanding of the effectiveness of malaria control tools, which is very important as Cambodia had engaged for malaria elimination by 2025.

In this context, **the general objective** of this thesis is to implement an existing assay based on the Luminex-technology for the detection of Abs against malaria parasites in blood samples of the baseline survey, to make it possible to detect 20 or more malaria specific Abs at the same time instead of each specific Ab separately such as in the ELISA.

Specific objectives:

1. Implementation and optimization of the assay.
    - Selection of the positive controls
    - Testing of the beads-coupling
    - Optimization of the numbers of beads/Ag/well and amount of volume for the detection Ab
    - Comparison of ΔMFI between monoplex and multiplex assay
    - Comparison of percent positivity between ELISA and multiplex assay
2. Screening blood samples of the baseline survey for detection of anti-malarial Abs.
    - Quality control of the high positive control and calculation of the cut-off values for sero-positivity per Ag.
    - Comparison of the used serological markers for their sero-prevalence and the levels of specific Ab responses.
    - Comparison between the numbers of Ags recognized by PCR positive and negative individuals.
    - Comparison of the percent positivity for each serological marker between Plasmodium PCR positive and negative samples.
    - Comparison of the proportion of sero-positivity of each serological marker between different groups in the baseline survey (e.g. age, gender and districts).

3 Material and Methods

Figure 7 shows an overview of the practical steps performed during this research. The steps will be explained in the following paragraphs.

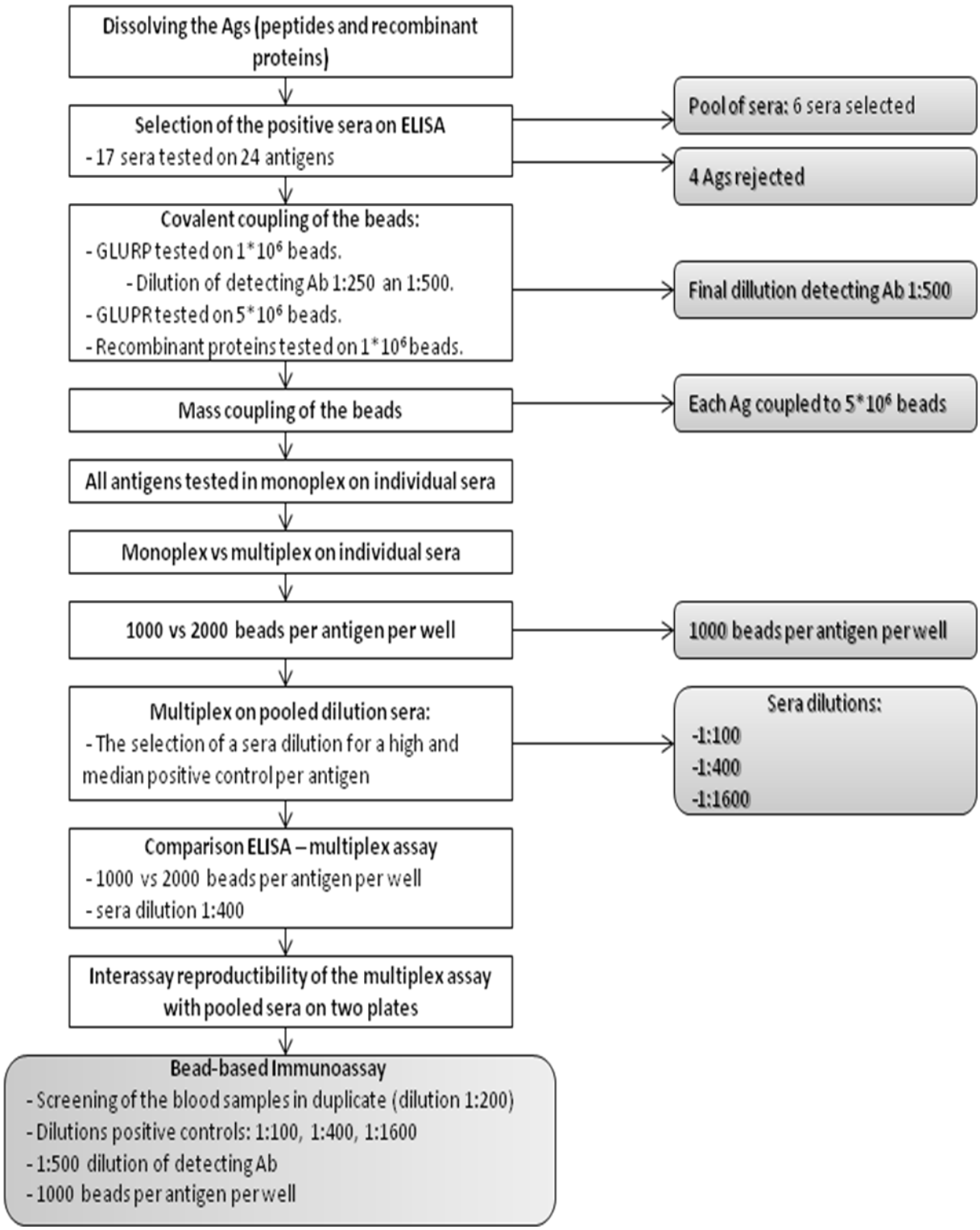


Figure 7: Overview of the practical steps.

3.1 Antigens

Various Ags are used for the multiplex bead assay. Nineteen peptides (Table 3) and five Recombinant proteins (Table 4) that are *Plasmodium* specific are synthesized with an added N-terminal cysteine residue and covalently coupled with BSA (bovine serum albumin, Sigma-Aldrich, St. Louis, USA) [27] by GeneCust Europe (Dudelange, Luxembourg) and stored in aliquots at -20°C. Two *Anopheles* salivary Ags (Table 3) were designed by bioinformatics [27] and also included in the panel. The selection of the peptides is based on the paper of Ambrosino et al [27] and the selection of the recombinant proteins is based on the availability at the 'Institut Pasteur' (IP) Paris.

Peptides	Sequence (N-teminal to C-terminal)	g/mol	life-cycle stages	*Plasmodium* species	
Lsa1-41	LAKEKLQEQQSDLEQERLAKEKLQEQQSDLEQERLAKEKEKLQC	5297,97	Liver stage	*P. falciparum*	[27, 31]
Lsa1J	ERRAKEKLQEQQSDLEQRKADTKKC	3046,43	Liver stage	*P. falciparum*	[27, 60]
Lsa3NR2	VLEESQVNDDIFNSLVKSVQQEQQHNVC	3230,53	Liver stage	*P. falciparum*	[27, 60]
Lsa3RE	VESVAPSVEESVAPSVEESVAENVEESVC	2991,20	Liver stage	*P. falciparum*	[27, 60]
Glurp	EDKNEKGQHEIVEVEEILC	2241,47	Trophozoite	*P. falciparum*	[27, 60]
GlurpP3	EPLEPFPTQIHKDYKC	1945,23	Trophozoite	*P. falciparum*	[27, 60]
Salsa1	SAEKKDEKEASEQGEESHKKENSQESAC	3123,24	Sporozoite & Liver stage	*P. falciparum*	[27, 60]
Salsa2	NGKDDVKEEKKTNEKKDDGKTDKVQEKVLEKSPKC	4019,52	Sporozoite & Liver stage	*P. falciparum*	[27, 60]
Trap1	DRYIPYSPDRYIPYSPDRYIPYSPC	3097,47	Sporozoite	*P. falciparum*	[27, 60]
Trap2	CHPSDGKCNCHPSDGKCNC	2005,21	Sporozoite	*P. falciparum*	[27, 60]
StarpR	STDNNNTKTISTDNNNTKTIC	2299,42	Sporozoite	*P. falciparum*	[27, 60]
CSP	NANPNANPNANPNANPNVDPNVDPC	2557,67	Sporozoite	*P. falciparum*	[27, 60]
SR11.1	EEVVEELIEEVIPEELVLC	2213,54	Sporozoite & Liver stage	*P. falciparum*	[27, 60]
Saliv1	EKVWVDRDNVYCGHLDCTRVATFC	2830,22	Salivary gland proteins	*An. gambiae*	[27]
Saliv2	ATFKGERFCTLCDTRHFCECKETREPLC	3324,84	Salivary gland proteins	*An. gambiae*	[27]
PvVK210CSP	DGQPAGDRAAGQPAGDRADGQPAGDRADGQPAGC	3206,30	Sporozoite	*P. vivax*	[60]
PvVK247CSP	ANGAGNQPGANGAGNQPGANGAGNQPGANGAGNC	2905,95	Sporozoite	*P. vivax*	[60]
Pv-likeCSP	APGANQEGGAAAPGANQEGGAAAPGANQEGGAAC	2892,99	Sporozoite	*P. vivax*	[60]
PmCSP	GNAAGNAAGNDAGNAAGNAAGNAAGNAAGNAAC	2358,37	Sporozoite	*P. malariae*	[60]

Table 3: Sequences of nineteen peptides used in this study.

Recombinant	life-cycle	*Plasmodium*	
PfMSP1-19	Merozoite	*P. falciparum*	[60]
PvMSP1-19	Merozoite	*P. vivax*	[60]
PfGLURP-R2	Merozoite	*P. falciparum*	[60]
PvDBP	Merozoite	*P. vivax*	[30]
Pf13	Sporozoite	*P. falciparum*	[61]

Table 4: The five recombinant proteins used in this study.

First all peptides are dissolved in 1 mL dH_2O (distilled water) except peptides GLURP-P3 and Lsa3-RE which are dissolved in 2 mL dH_2O due to lower solubility (Table 5). Peptides Pm CSP, SR11.1, SALIV 1, SALIV 2, TRAP 1, GLURP, LSA1-NR2 and STARP-R did not dissolve by only using dH_2O. Therefore, we added 40µL of NH_4OH (ammonium hydroxide) to dissolve these peptides (Table 5) [62].

Peptides (1mL)	pI	Hydropathicity	Hydrophilic/ hydrophobic	Cysteine/ Methionine	dH_2O (mL)	NH_4OH (µL)
Pm CSP	3.8	-0.158	Hydrophilic	Cys & Met	1	40
Lsa1-41	4.88	-1.661	Hydrophilic	Cys & Met	1	-
Lsa1-J	9.05	-2.232	Hydrophilic	Cys & Met	1	-
Lsa3-NR2	4.17	-0.593	Hydrophilic	Cys & Met	1	40
Lsa3-RE (2mL)	3.36	0.069	Hydrophobic	Cys & Met	2	-
Glurp	4.3	-1.011	Hydrophilic	Cys & Met	1	40
Glurp P3 (2mL)	5.45	-1.137	Hydrophilic	Cys & Met	2	-
Salsa 1	4.72	-2.186	Hydrophilic	Cys & Met	1	-
Salsa 2	8.05	-2.117	Hydrophilic	Cys & Met	1	-
Trap 1	6.03	-1.112	Hydrophilic	Cys & Met	1	40
Trap 2	6.87	-1.121	Hydrophilic	Cys & Met	1	-
Starp R	5.68	-1.433	Hydrophilic	Cys & Met	1	40
CSP	3.56	-1.34	Hydrophilic	Cys & Met	1	-
SR11.1	3.36	0.532	Hydrophobic	Cys & Met	1	40
Saliv 1	5.43	-0.204	Hydrophilic	Cys & Met	1	40
Saliv 2	6.77	-0.589	Hydrophilic	Cys & Met	1	40
Pv-like-CSP	3.67	-0.465	Hydrophilic	Cys & Met	1	-
PvVK210 CSP	4.11	-0.212	Hydrophilic	Cys & Met	1	-
PvVk247 CSP	5.56	-0.906	Hydrophilic	Cys & Met	1	-

Table 5: information for dissolving the peptides [63].

This table shows the amount of ammonium hydroxide (NH_4OH) added for dissolving the peptides. Peptides which are hydrophilic are usually dissolved in dH_2O and if the peptides not dissolve a basic solution is added to the peptides. If the peptides are Hydrophobic TFA is usually added to dissolve the peptides.

3.2 ELISA used for positive sera selection

The ELISA is used to select the positive controls by screening seventeen random selected serum samples (Table 6 and Table 7) from malaria positive patients from the Ratanakiri province for the presence of Abs to the different Ags in order to identify serum samples that can be used as positive controls in the Luminex assay. The sera from the malaria positive patients are tested by PCR and confirm that those patients are parasite carriers at that certain moment. As the ITM has experience with using Pf GLURP-R2 recombinant protein in the ELISA and possesses a control serum (REF_P_sera; pooled sera from five *P.falciparum* or *P.vivax* infected patients), made by Cook et al [38], that is found positive for Pf GLURP-R2. This combination of Ag and serum is used as a control for all the ELISA plates.

Serum ID *P. falciparum*	Age (years)	Sex	Parasite load/µL	Serum ID *P. vivax*	Age (years)	Sex	Parasite load/µL	Serum ID *P. malariae*	Species	Age (years)	Sex
5035	39	Male	63554	C1 0006	12	Female	10520	1678	Pm		
5036	47	Male	161647	C1 0009	21	Male	29629	3154/0038/09	Pm	4	Male
5049	58	Male	156000	C1 0021	22	Female	12761	3174/0018/02	Pm	20	Female
5051	40	Female	109650	C1 0026	7	Female	14960	3215/0013/04	PfPm	22	Female
5072	17	Male	5019	C1 0028	8	Female	22727	3300/0035/01	PfPm	14	Male
5078	23	Male	38685	C1 0044	11	Male	30534	3317/0017/02	PfPm	37	Female
5100	46	Male	31651	C1 0052	31	Male	24504	3324/0120/01	Pm	38	Male
5138	32	Female	43700	C1 0056	8	Female	42909	3435/0009/07	Pm	15	Female
5145	30	Male	227524	C1 0059	39	Male	26666				
5191	44	Male	88171	C1 0069	20	Male	25440				
5208	38	Male	51406	C1 0078	39	Male	25670				
5248	16	Male	12754	C1 0082	30	Female	12739				
5274	19	Male	11700	C1 0090	22	Female	11356				
5286	31	Male	510	C1 0095	8	Male	16283				
5287	19	Male	28363	C1 0122	12	Male	16640				
5288	18	Male	4960	C1 0139	16	Male	13959				
5373	19	Male	525000	C1 0185	23	Female	35809				

Table 6: serum information of the seventeen random selected sera for *P. falciparum* and *P. vivax* and eight random selected sera for *P. malariae*.

Serum ID (*P. falciparum*)	Age (years)	Sex	Parasite load/µL	Serum ID (*P. vivax*)	Age (years)	Sex	Parasite load/µL
5005	24	Male	42101	4872	8	Male	26000
5006	26	Male	24230	4887	42	Male	18000
5016	18	Male	12830	4983	10	Male	24000
5017	29	Male	20808	5009	18	Male	26000
5019	32	Male	41375	5013	25	Male	22000
5034	20	Male	11984	5064	26	Male	30000
5104	24	Male	305600	5084	31	Male	20000
5118	24	Male	99780	5095	44	Female	32000
5168	24	Male	40192	5114	20	Male	32000
5209	18	Male	104000	5116	23	Male	16000
5243	32	Male	82540	5166	37	Male	95000
5244	35	Male	11783	5246	18	Male	20000
5269	24	Male	21320	5502	15	Female	30000
5312	25	Male	143500	5505	28	Male	24000
5328	14	Male	47996	5655	29	Male	20000
5333	57	Female	34754	5709	19	Male	24000
5334	50	Male	72286	5719	16	Male	26000

Table 7: Serum information.

This sera is used when the first 17 random selected sera did not gave optimal results within the ELISA and when there was no positive control for one of the Ags.

All twenty-four Ags and seventeen serum samples per plate are tested on the ELISA (procedure in ANNEX 1) and compared to the REF_P_sera and Pf GLURP-R2 recombinant protein (control) [38]. The 96-well plates are coated with 200 µL PfGLURP-R2 (control; 2µg/ml) per well and 200 µL test-Ag (2µg/ml) per well by overnight incubation at 4°C (Figure 1 in ANNEX 1). The 96-well plates are emptied and washed 3 times with 300 µL of PBS-Tween (0.05% Tween in PBS 0.01M pH 7.4) per well. Then 200 µL of serum sample diluted at 1:400 in PBS-Blotto to serve as positive control is added in every well according to the plate configuration in the ELISA SOP (Figure 2 in ANNEX 1) and for the blanco 200 µL of PBS-Blotto (0.01 M pH 7.4 ± 2) is added. This is incubated for 1 hour at ambient temperature. The 96-well plates are emptied and washed 3 times with 300 µL of PBS-Tween (0.05% Tween in PBS 0.01M pH 7.4) per well. Then 200 µL of conjugate (1:50000 in PBS-Tween) is added to every well and incubated for 1 hour at ambient temperature. The 96-well plates are emptied and washed 5 times with 300 µL of PBS-Tween (0.05%) per well. At last 200 µL ABTS-substrate is added to every well on the plates and incubated for 1 hour at ambient temperature. Optical densities (OD) are measured at 415 nm (OD_{415}). All samples are analyzed in duplicate. The well-plates are divided in 2 sections (coated-site and non-coated-site) to calculate the corrected OD-values by using the background levels ($COD=OD_{coated\ wells} - OD_{non\text{-}coated\ wells}$). The percent positivity (PP) of each sample is calculated by using the positive control serum (GLURP R2) OD as 100% to ensure that the sample results are standardized across the ELISA plates (ANNEX 1) [38]. With every run, negatives are included as well. For negative controls, blood of a person who has never been in contact with malaria is used.

After testing all twenty-four Ags on the seventeen random selected serum samples the strongest sera are selected for each Ag to construct a pool serum. The Ags that gave too low results to select a good positive control serum are rejected from this research (in total four Ags are rejected). The strongest selected sera for the remaining twenty Ags are pooled together in a 1:100 dilution (500µL of each sera + 47mL PBS-CR). Then, the pooled sera are tested on the ELISA for all remaining Ags on a dilution of 1:400. The combination of these sera is used as positive controls for all *P. falciparum* and *P. vivax* Ags in the Luminex immunoassay.

3.3 Covalent coupling of Ags to the beads/microspheres

Covalent coupling of the beads (MagPlex microspheres, Luminex Corp., Austin, TX, USA) is carried out as described by Ambrosino et al [27] and the Luminex Corporation (Figure 9) [64, 65].

In total, twenty Ags were selected for this research and covalently coupled to twenty different beads, each labeled with a different color code based on a specific ration of two fluorochromes that can be detected by the MAGPIX apparatus [65]. Every bead contains about 10^8 COOH-groups at its surface. During the coupling process, about 10^6 COOH-groups will couple randomly with the added Ags [39]. Coupling of the beads is achieved through a carbodiimide reaction between carboxyl groups on the surface of the beads and primary amino groups on the proteins (Figure 8).
The coupling involves 2 major steps. It starts with the activation of the carbocyl (COOH) groups with EDC (1-Ethyl-3-[dimethylaminopropyl] carbodiimide hydrochloride) to form an unstable reactive O-acylisourea intermediate. This forms a more stable ester when Sulfo-NHS (N-hydroxysulfosuccinimide) is added to the beads. Then the ester reacts with the primary amines (NH_2 groups) on the Ags by forming a stable covalent linkage as described in the protocol from Luminex Corporation (Austin, TX, USA) [64, 65].

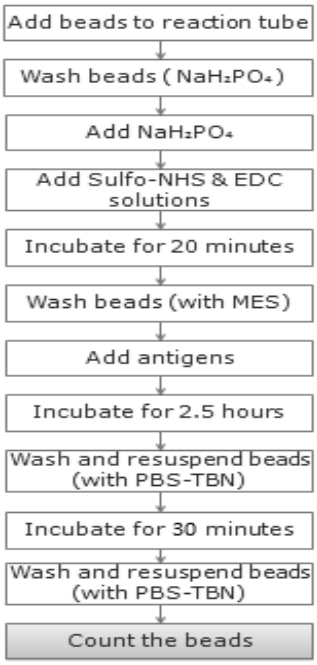


Figure 9: Model of the coupling protocol [64].

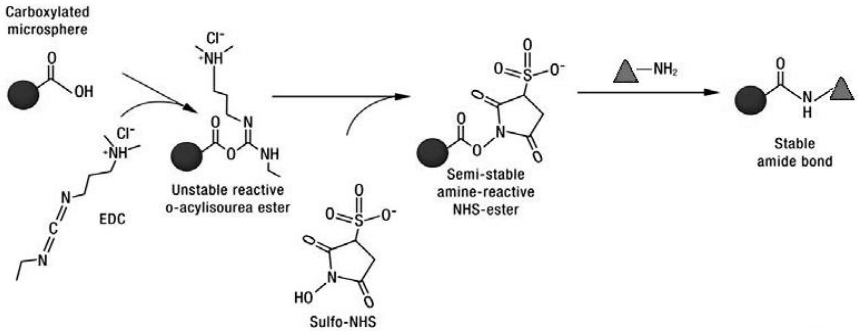


Figure 8: Coupling of the beads between the carboxyl- and amino groups [64].

The last step within the bead coupling process is counting of the beads performed on the hemocytometer (within a 4x4 grid (0.1µL)) to check if there is a loss of beads [39]. When an amount of $5x10^6$ beads is used and resuspended in 125µL PBS (Wash buffer) and 125µL CR (Charles River, MFIA: Multiplexed Fluorescence ImmunoAssay) a maximal amount of 200 beads has to be counted in the hemocytometer.

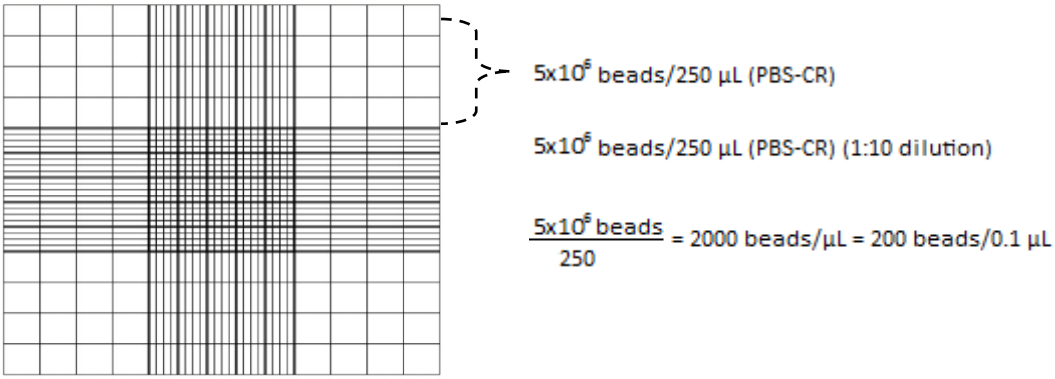


Figure 10: Hemocytometer [64].

3.4 Testing of the Antigen-Bead Coupling

Testing of the Antigen-Bead coupling is first performed on GLURP (peptide) by using a small amount of beads (minimum of $1x10^6$ beads). The Ag amount is used as described in Table 1 and Table 2 in ANNEX 2). For testing the Ag binding to the beads, the positive control selected for this Ag and the negative control are used. Therefore, 8 serial dilutions (1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400; 1:12800; 1:25600) of the appropriate positive control and 1 negative control for GLURP are tested by using a titration (e.g. 1:250 and 1:500) of the detection Ab (ANNEX 2). This step is performed following the protocol of covalent coupling of the Ags to the beads and the immunoassay protocol (ANNEX 2) [27, 39]. The next step is testing GLURP on a large amount of beads ($5x10^6$ beads) following the same protocol as described for the small amount of beads. After confirmation that the coupling of the beads was performed well on GLURP, all other peptides are tested on the large amount of beads, because all peptides are linked to BSA and are expected to have similar binding capabilities. The strongest sera selected for each individual Ag is used as the positive control (ANNEX 3). Then, the Antigen-Bead coupling is tested in small scale for the recombinant proteins following the same protocol as performed on GLURP. Individual testing of the recombinant proteins is essential, as it is not known if the recombinant proteins bind similarly to the Ags as the peptides, because of the differences in their sequence.

For the selection of the serum dilutions of the positive control pool, which will be used in the Immunoassay, the dilutions are determined for each Ag for a strong positive reaction (100%) and the corresponding 50% reaction and are used for further analysis (Figure 11).

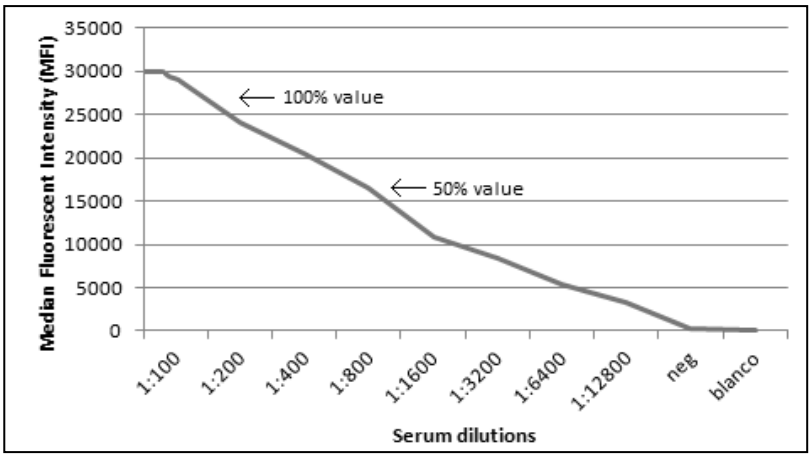


Figure 11: Example of the titration curve. The 100% value is the value of the strong positive control and the 50% value is the value of the low positive control. These are used as a control in the final sample survey.

After selecting the strong positive and medium positive dilutions of the control pooled sera the dilutions will be tested in the multiplex assay. Then, the percent positivity of the dilution 1:400 will be compared to those obtained with the ELISA.

3.5 Comparison between the monoplex and multiplex assay

A comparison between the monoplex assay and the multiplex assay is performed for all the Ab responses. For the monoplex assay each Ag is tested once on its strongest serum (chosen from the selected positive sera) (ANNEX 3). For the multiplex assay a microsphere working mixture is made and tested once on the same individual sera. Serum dilutions (1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400; 1:12800; 1:25600) are made from the individual and pooled sera. This step is performed following the protocol of the covalent coupling of the Ags to the beads and the immunoassay protocol (ANNEX 2) [27]. The results of the monoplex and multiplex assay are expressed in ΔMFI (corrected MFI), defined by the following formula: $\Delta MFI = MFI_{Ag} - MFI_{BSA}$. The MFI_{Ag} is the median value of the individual bead coupled Ags and MFI_{BSA} is the background MFI that represents for the non-coupled beads [59].

3.6 Inter-assay reproducibility of the multiplex assay

The accuracy of the multiplex immunoassay is defined as the reproducibility between assays and is important to guarantee that the results obtained with the immunoassay throughout the whole research are accurate and reproducible from one to another assay. The inter-assay reproducibility give a warranty that the results obtained within two different plates are indeed reproducible. Therefore, a multiplex assay (20 Ags) will be performed on two plates on the pooled sera dilution series (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600) on two different days to see if there is a similarity in the repeatability between the two plates.

3.7 Difference between the amount of beads per well

The difference between the amount of beads per well is tested for an amount of 2000 beads/well and 1000 beads/well. This test is performed to see if there is a difference between the use of 1000 beads/well or 2000 beads/well. If the results are similar an amount of 1000 beads/well will be used. For determining the amount of beads per well a microsphere working mixture is made and tested in triplicate for 2000 beads/well (50µL) and for 1000 beads/well (25µL) on the pool of sera following the 8 serial dilutions. The results are expressed in ΔMFI and compare to each other by using a standard curve [66]. Furthermore, the difference between the use of 50µL of detection Ab and 100µL of detection Ab, is tested at this stage. From the results the mean and standard deviation are calculated to give an indication of the spread of the data around the mean. From the standard deviation and the mean the relative standard deviation (RSD) is calculated and expressed as a percentage. The higher the percentage the higher the variability in the data set.
To perform this test the protocol of the covalent coupling of the Ags to the beads and the immunoassay protocol (ANNEX 2) is followed [27].

3.8 Bead-based Immunoassay

After coupling of the Ags to the beads/microspheres, the immunoassay will be prepared as described by Khaireh et al [58], Ambrosino et al [27] and the Luminex Corporation [39, 65].

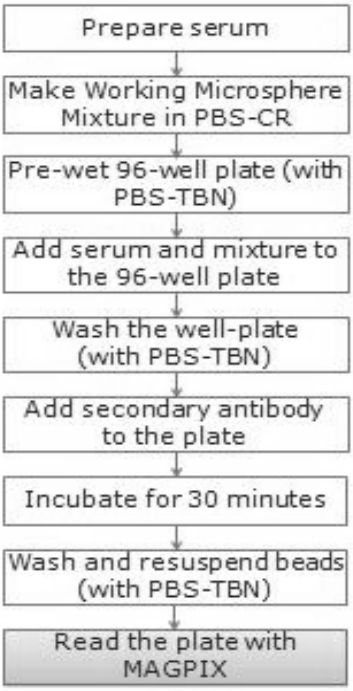


Figure 13: Model of the Immunoassay protocol [27, 39, 58, 65].

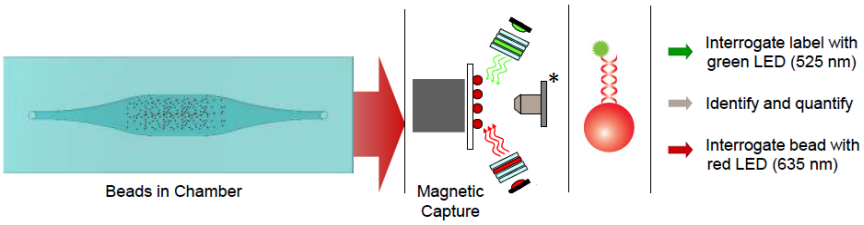


Figure 12: MAGPIX readout system [41].

1. Paramagnetic beads are sent to the chamber were they are agitated and aspirated.

2. The magnet is switched on and will pull the beads out of the suspension onto the back plane of the chamber and hold the beads in place.

3. The Red LEDs are turned on and an image is taken through the band pass filter of the filter wheel (*) followed by a second image. Red LEDs are used to distinguish the different beads by detecting the dye ratio.

4. Then, the Red LEDs are turned off and the Green LEDs are turned on. A third image is then taken through the band pass filter. The Green LEDs are used to detect the detection Ab (detection PE-labeled- Ab)

The serum is prepared from the blood spot filter papers as mentioned above. The next step is preparation of the Microsphere Working Mixture in PBS and CR diluents (Charles River Laboratories

Inc, MA, USA) at a final concentration of 1000 beads/µL (25µL/well) per peptide [27, 58, 59, 65], followed by preparation of the 96-well roundbottom plates (Figure 130. To obtain optimal results, all plates with the final samples are prepared in duplicate.
The plate is read with the MAGPIX (Luminex Corporation) that is set for reading 25µL/well with a minimum of 400 beads per spectral address (Figure 12). The MAGPIX will give two outputs of data from an acquisition: MFI (median fluorescent intensity) value and the bead count [27, 41, 59]. The median is used instead of the mean, because the median is less sensitive to outliers that are created by possible bead-carryover of about 4% from a previous well [41]. The results are expressed in ΔMFI value (corrected MFI, see paragraph 3.6) and converted into a percent positivity, achieved by the use of a predetermined dilution of the positive control as the 100% value (Figure 11) [59].

3.9 Serum samples

Within the repellent project, 98 clusters of villages in Ratanakiri province (eastern Cambodia) participate. Of these, half have access to repellents and bednets (intervention clusters), the other half only has access to bednets (control clusters). At regular times, surveys are organized, taking blood samples from randomly chosen people in these 98 clusters of villages. Per cluster, samples are collected from an average of 50 inhabitants chosen at random. Thus, in total about 5.000 samples are collected by finger prick on a filter paper at the start of the rainy season in April, and six month later at the end of the rainy season in October-November. All of these blood samples were analyzed by PCR for the presence of the Plasmodium parasite as primary outcome. A secondary outcome of the project is based on serology, by detecting Abs against *Plasmodium* Ags. For this master thesis a randomly selected amount of about 2.000 collected bloodspot filter papers from different ages from the baseline survey of the project are used for anti-*P. falciparum*, -*P. vivax,* -*P.malariae* and -*Anopheles saliva* Ab detection. For the Immunoassay, the blood spot filter papers are prepared by punching out 2 discs of 4mm (diameter) and eluted in 160 µL of PBS-1% BSA-0.15% Tween (dilution of 1:40). This is eluted over night at room temperature on a plate shaker. Then the concentration is adjusted to a dilution of 1:200 with PBS-CR (Phosphate buffered saline-Charles River).

3.10 Data analysis

In order to select the positive serum for the sera pool, the percent positivity (PP) of each sample is calculated in Excel and displayed using Graph Pad Prism® (Graph Pad, San Diego, USA) version 6, by using the positive control serum (Pf GLURP R2) OD as a 100% value. Then, data obtained with testing the beads-coupling, the comparison between the monoplex and multiplex and the 1000 and 2000 beads/Ag/well are incorporated using Excel. The Spearman Rank correlation test (non-parametric) is used to measure the relation between two variables. This test was appropriate, since only relative values (PP) will be used in the final assay, thus decreasing the importance of differences in absolute values of MFI.

Data obtained in the final bead-based immunoassay were analyzed with different statistical software packages (SPSS version 17 (SPSS Inc., Chicago, Illinois), preprogrammed R software (WU, Wien, Austria) version 2.15.2 and preprogrammed STATA software (Statacorp., Texas, USA) version 12). To calculate the correlations between continuous values in the testing phase the Spearman Rank correlation test was used.

After implementation and optimization of the multiplex assay, screening of the blood samples of the baseline survey for detection of anti-malaria Abs is performed. Figure 14 presents the different steps performed for validation of the high positive control (serum pool see Table 12 in paragraph 4.5) and calculation of the cut-off value for determining the sero-positivity per Ag.

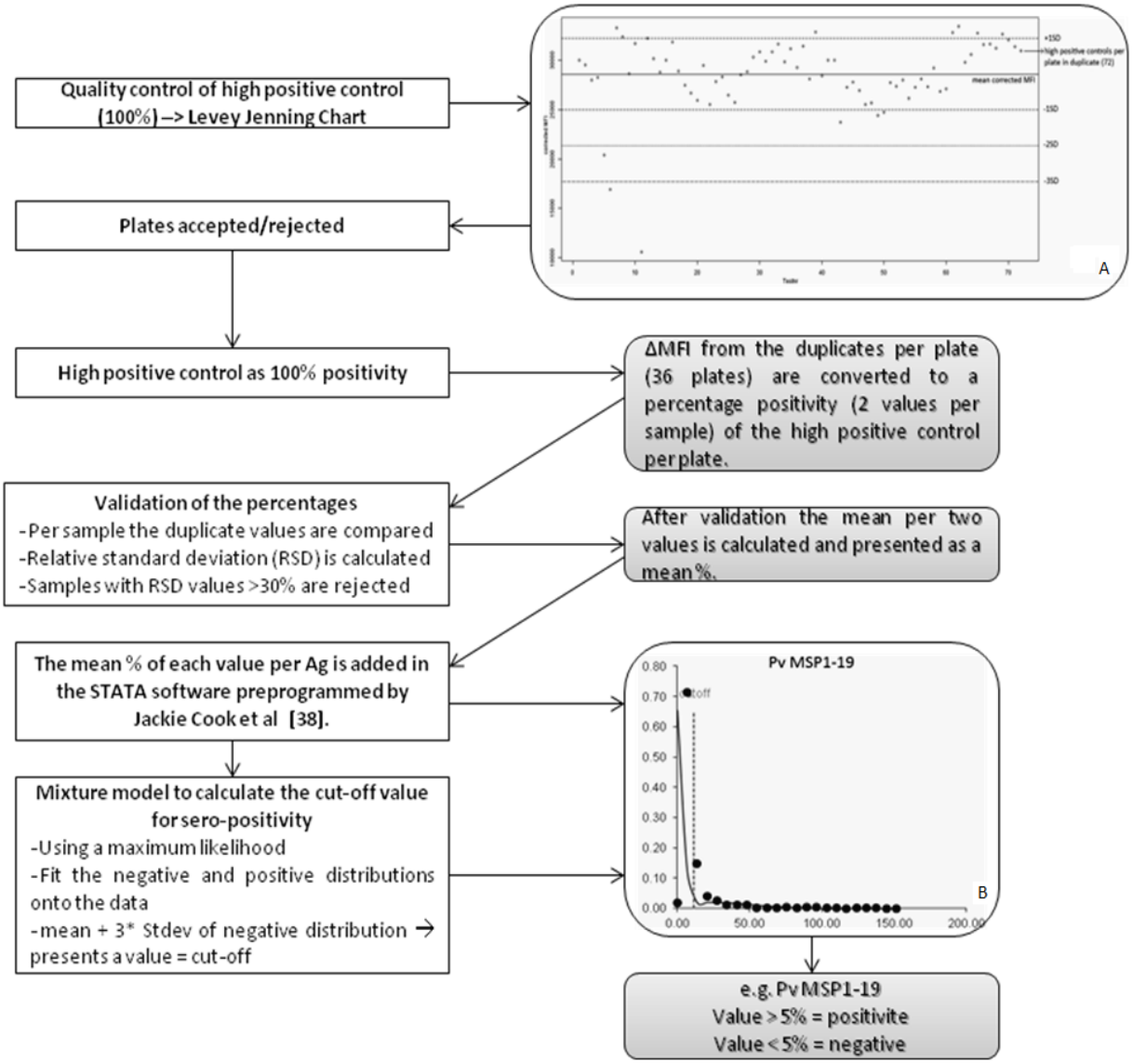


Figure 14: Workflow of the steps performed to validate the high positive controls and to determine the cut-off per Ag. Graph (A) is an example of a Levey Jenning Chart used for Quality control of the high positive controls (100%) and graph (B) is an example of a Gaussian curve to determine the cut-off value for sero-positivity for Pv MSP1-19.

First, a quality control of the high positive controls is done per Ag. The quality control of the medium positive controls is not within the scope of this thesis, but can be done in a later stage. The serum dilution (1:100 or 1:400) used to determine the high positive control (100%) varies from Ag to Ag (see table Table 12 in paragraph 4.5). The Levey Jenning chart in (Figure 14.A) shows the seventy-two different high positive controls for one Ag and is made for all twenty different Ags. Per plate (in a 96 round bottom well plate) 2 wells are used for the high positive control and each plate (18 plates) is made in duplicate. The dotted horizontal line stands for the ΔMFI (mean) for that Ag. The line above the dotted horizontal line represent the upper limit (+1SD; +2SD and +3SD are not shown in the example graph) and the lines under the dotted horizontal line represent the lower limits (-1SD, -2SD and -3SD). The dots that fall within the +2SD and -2SD limits are accepted values. The plates of the dots that do not fall within the limits are not accepted and are rejected (whole plate 2a & 2b and one of the two high positive controls from plate 3a). After removing whole plates 2a and 2b are from the 1440 screened blood samples there still are 1360 samples left and used for further validation of the samples (described in the next steps). The Levey Jenning chart shows that there are fluctuations

between the different dots and plates, which are possibly due to daily variations in experiments, eg temperature, incubation times, pipetting differences etc.
After quality control of the high positive controls, the high positive control will be set as a 100% value per Ag for validation of the samples (made in duplicate). For the Ags (SR11.1, SALIV2, PmCSP, Pv like CSP and PvVK210 CSP) that gave too low results for the high positive control, the high positive control of GLURP (peptide) is used. Therefore, the ΔMFI from the duplicates per plate (36 plates) are converted to a percentage (two values per sample). The percentages are validated per sample, by calculating the relative standard deviation (RSD). Samples with a RSD > 30% are rejected for further analysis. After validation of the samples the mean (presented as a mean %) of the two separated values are calculated per sample per Ag. The cut-off value for the positivity is generated by adding the mean % of each value per Ag in the STATA software (Statacorp, Texas, USA) preprogrammed mixture model by Cook et al [38]. As explained by Cook et al [38] this mixture model uses maximum likelihood to fit the negative and positive distribution onto the data, which is based on a "simple reversible catalytic conversion model". Once the catalytic conversion model has been solved the graph shown in Figure 14 will show the spread of data by creating a Gaussian curve (Figure 14.B) for the negative and positive data and the selected cut-off value, based on the mean + 3*standard deviation of the negative distribution (=cut-off). In Figure 14 an example of the cut-off value is calculated for Pv MSP1-19, of which the value of sero-positivity is determined as a 5% cut-off value. When the samples are >5% they are positive and when the samples are <5% they are negative.

Furthermore, non-parametric statistical analyses were performed to test the difference at the baseline survey in the proportions of Ab-positive individuals between different groups (age, gender, districts) [27, 59]. The non-parametric tests used to analyse the proportions of sero-positivity are the Mann-Whitney U test (=2 groups) and Kruskal-Wallis test (≥3 groups). Differences are statistically significant at a $p<0.05$ [27, 59].
The 95% confidence intervals are calculated for the sero-positivity per Ag, per age group and per gender to indicate the reliability of an estimation [59].

4 Results

4.1 Positive sera and Ag selection on the ELISA

Seventeen malaria positive sera from Cambodia were tested on ELISA for the selection of positive controls to assess the Luminex assay. Therefore, all twenty-four Ags were each tested on a different plate for the seventeen sera (Figure 15 and Table 8). All ODs were compared to the OD value of REF_P_sera for Pf GLURP R2 (100%) and compared to percentages. From each Ag the strongest positive sera (5051, 5333, 5208, 5248, C1 0026 and C1 0082) were selected, pooled and used as a positive control for interplate variation. Four Ags (Saliv1, TRAP1, TRAP2 and PvVK247-CSP) are excluded from this research, because these gave too low results to select a good positive control serum.

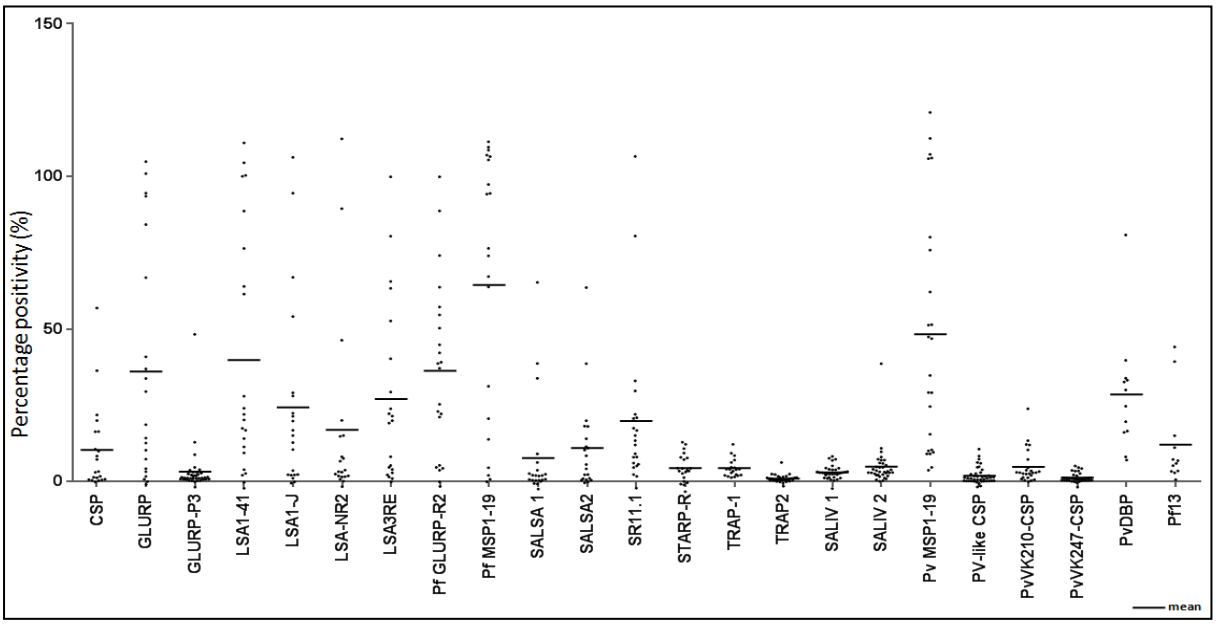


Figure 15: All twenty-four Ags tested on 17 different sera for the selection of the strongest positive sera.

Antigens	Selected serum	Percent positivity of the individual serum (%)	Percent positivity of the pooled control (%)
GLURP	5051	105 %	145 %
LSA1-41	5051	111 %	127 %
LSA1-J	5051	106 %	117 %
GLURP-P3	5333	48 %	35 %
LSA3-NR2	5333	112 %	31 %
SALSA2	5333	63 %	48 %
Saliv2	5333	39 %	4 %
CSP	5208	57 %	47 %
Pf GLURP R2	5208	89 %	100 %
Pf MSP1-19	5208	111 %	151 %
LSA3-RE	5248	100 %	141 %
STARP-R	5248	11 %	5 %
Pf13	5248	44 %	25 %
PvDBP	C1 0026	81 %	76 %
SALSA1	C1 0082	65 %	107 %
SR11.1	C1 0082	107 %	110 %
Pv MSP1-19	C1 0082	121 %	115 %
Pv like CSP	C1 0082	8 %	0 %
PvVK210-CSP	C1 0082	12 %	-2 %
PmCSP	3215/0013/04	50 %	0 %
Pf GLURP R2	**REF_P_sera**		**100%**

Table 8: The Percent Positivity (PP) from the twenty selected Ags tested on the six selected positive sera individually and in pool (ANNEX 3) using the positive ELISA OD-values of REF_P_sera for Pf GLURP R2 as 100%.

Unfortunately for serum 3215/0013/04 which was positive for PmCSP only a limited amount was available. Therefore, this serum was not included in the pool, because the quantity required for the pool was not available. After selecting the positive controls for all twenty Ags, the 6 chosen sera were pooled and tested on the ELISA (ANNEX 3).

4.2 Testing of the beads-coupling

The Ag amount used for the bead coupling is calculated for a desired concentration of 4 µg/10^6 beads (ANNEX 2). BSA is coupled to the beads and used as background control [65] (ANNEX 2) to calculate the ΔMFI [59]. The pooled sera dilutions (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600) are used to determine the optimal serum dilution for the final multiplex assay.
As shown in Figure 16 the signal obtained by GLURP (1x10^6 beads) in the MAGPIX gave very good results. The ΔMFI values were high and reaching a maximum of 28797 for a detection Ab dilution of 1:250 and 22637 for a detection Ab dilution of 1:500. The detection Ab dilution of 1:500 is used for the rest of the MAGPIX testing, because the results are strong enough requiring less detection Ab. The signal obtained by GLURP (5x10^6 beads) in the MAGPIX gave also very good results. The ΔMFI values were high and reaching a maximum of 23700 for a detection Ab dilution of 1:500. We further use 5x10^6 beads for all BSA-linked peptides.

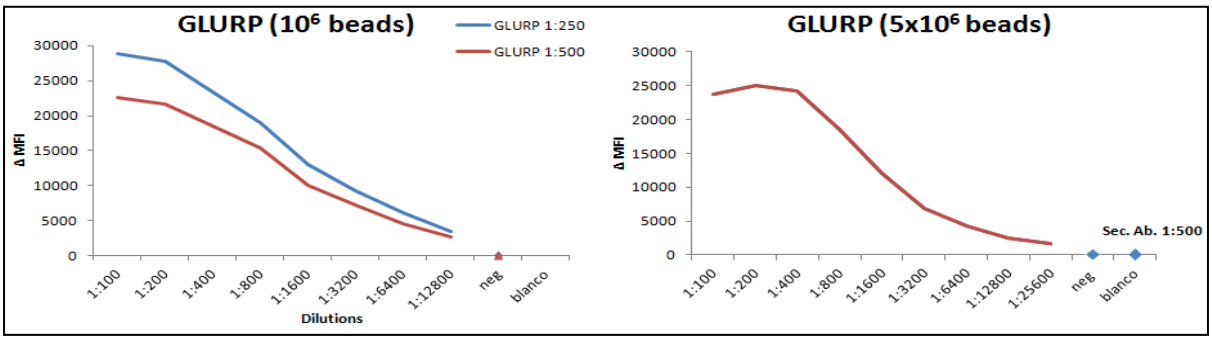


Figure 16: Small and large scale testing of the beads coupling with the peptides. The left graph shows the results of the small scale binding of GLURP (peptide) to the beads. In here two detection Ab dilutions were used 1:250 and 1:500. For both dilutions the MFI signal was very high with a maximum of 28797 (1:250) and 22637 (1:500). The right graph shows the results of the large scale binding with a detection Ab dilution of 1:500. The MFI signal had a maximum of 23700 (high).

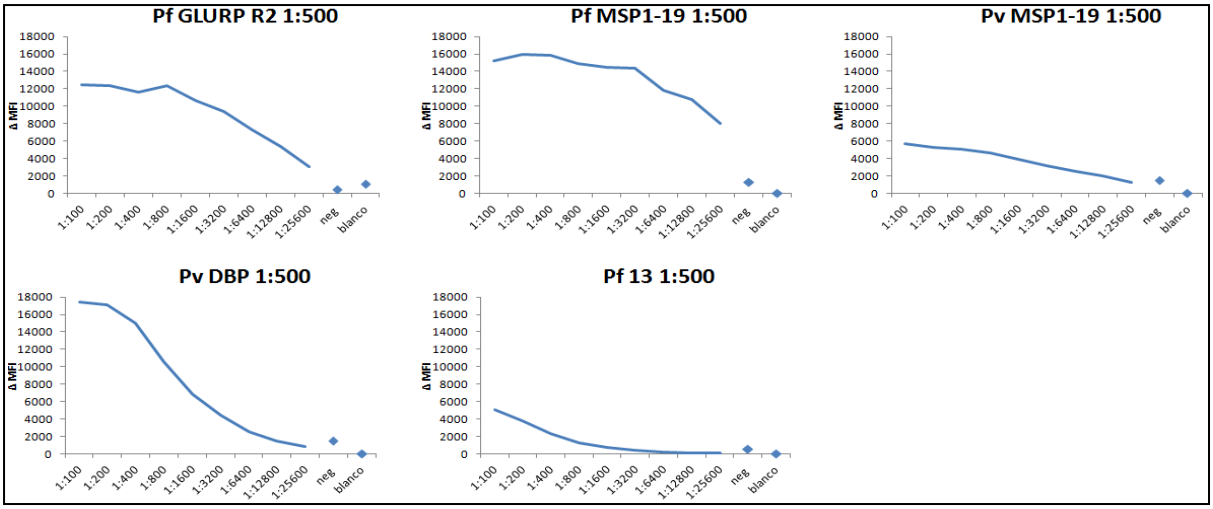


Figure 17: Small scale testing of the beads coupling with the recombinant proteins. A detecting Ab dilution of 1:500 is used. The MFI signal was high for Pf GLURP R2 (12506), Pv DBP (17389) and Pf MSP1-19 (15161) and low for Pf13 (5064) and Pv MSP1-19 (5723).

The ΔMFI signals from the recombinant proteins (1x10^6 beads) are shown in Figure 17. The ΔMFI values are good for Pf GLURP R2 (12506), Pv DBP (17389) and Pf MSP1-19 (15161). The ΔMFI values of Pf13 (5064) and Pv MSP1-19 (5723) were considered low compared to the other recombinant proteins on a detecting Ab dilution of 1:500.

4.3 Comparison between the monoplex and multiplex assays

A comparison between the monoplex assay and the multiplex assay is performed for all the Ab responses. Figure 18 shows the results (Δ MFI) obtained from the exposed individual sera (1:100 dilution) obtained from the Ratanakiri province which are incubated with single Ag-coated beads (blue bars) and with an equal amount of the 20 tested Ag-coated beads (red bars). According to the p-value ($p<0.001$) there is a significant correlation between the monoplex and multiplex assay. However, the results of some Ags (Lsa1-J, GLURP, Pf GLURP R2, Lsa3-RE, Pv DBP and Saliv2) give a stronger result in the multiplex assay compared with the monoplex assay.

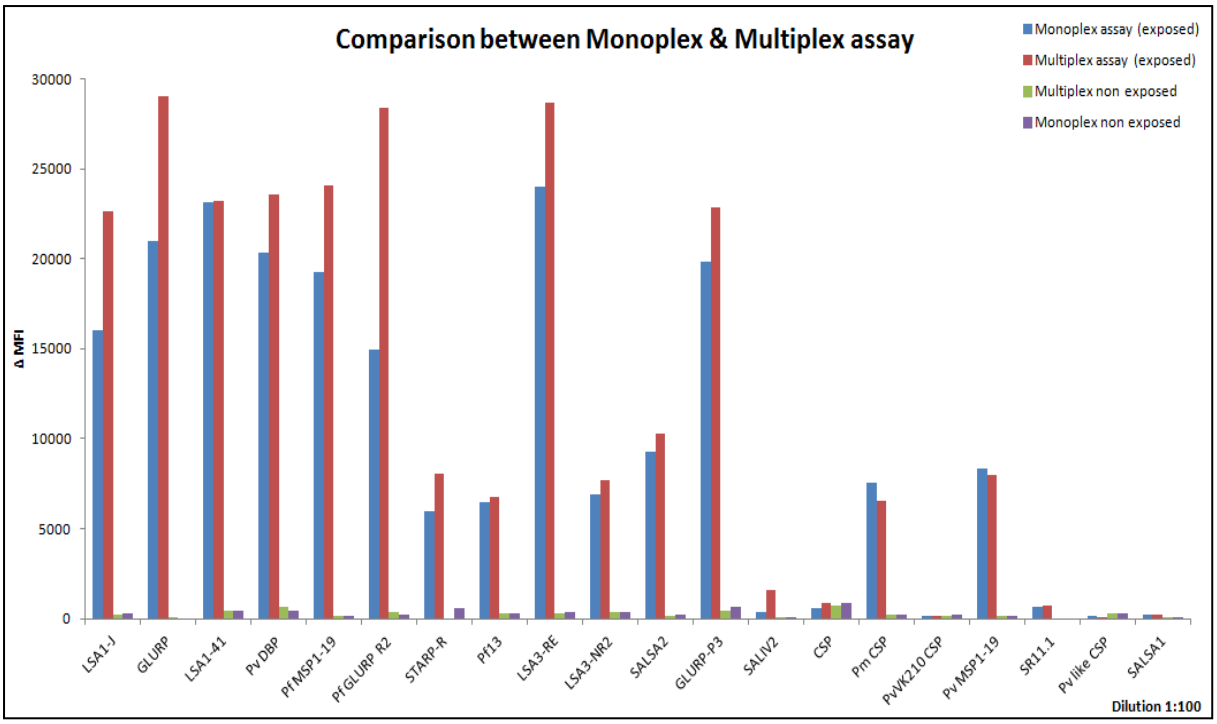


Figure 18: Comparison between exposed and unexposed individuals. The six selected positive sera were tested individually on the monoplex and multiplex assay and negative sera from an unexposed individual is tested. The obtained results were compared with each other at a dilution of 1:100 by the MAGPIX system. There is a clear correlation between the multiplex (red bars) and monoplex (blue bars) assays as confirmed by the correlation coefficient R^2 = 0.9285 and the Spearman rank correlation test p=5.39E-06. The multiplex- and monoplex non exposed are the results originating from the negative sera from an unexposed individual.

Figure 19 show results obtained from the individual sera per Ag. For some of the Ags (GLURP, LSA1-41, LSA1-J, Pf GLURP R2, Saliv2 and PvDBP) the multiplex assay gives a higher signal than the monoplex assay. The results in Table 9 show that there is a significant correlation between the monoplex and multiplex assay confirmed by the correlation coefficient (R^2) and the Spearman Rank correlation test.

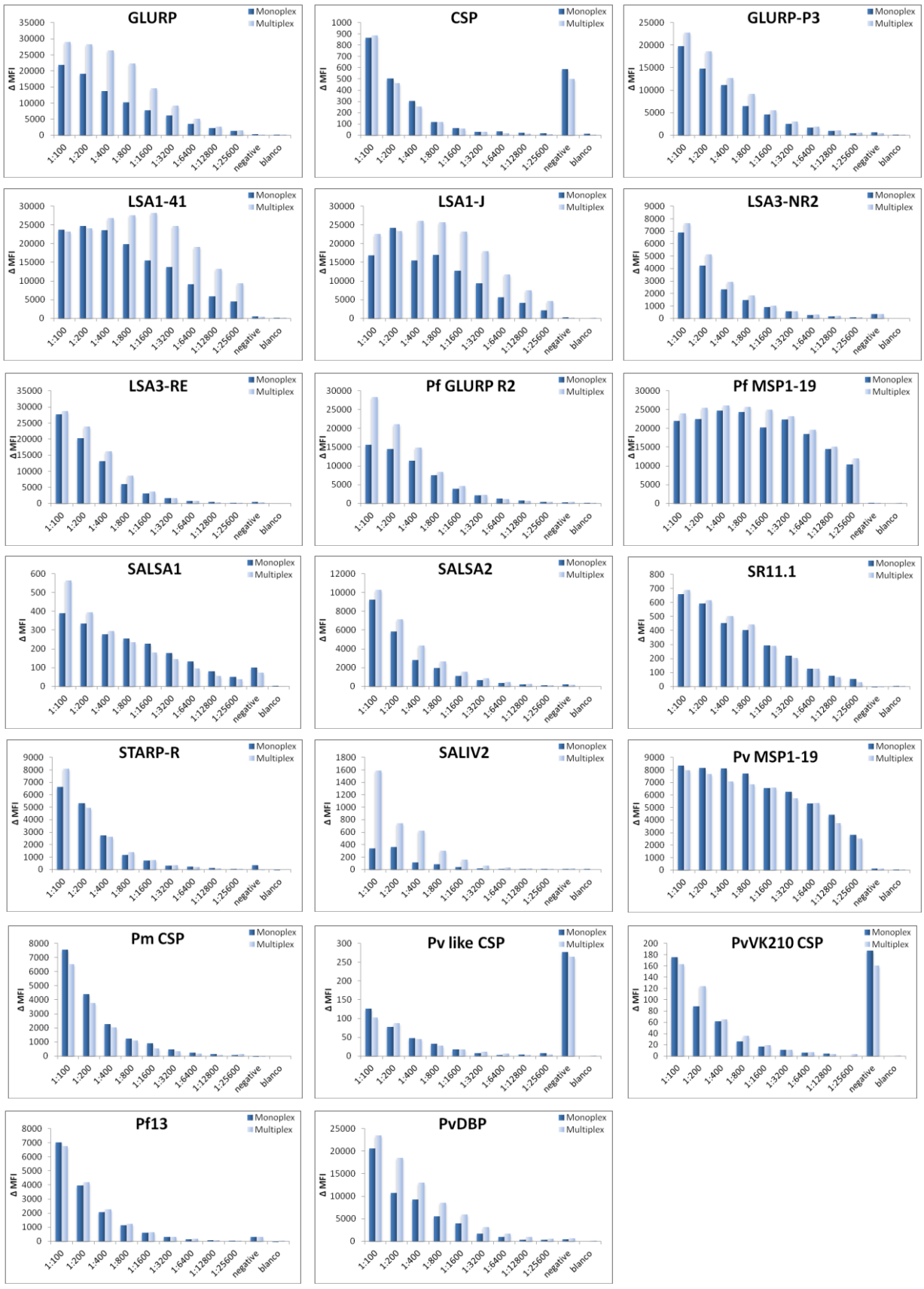


Figure 19: Comparison between the monoplex and multiplex assay for the individual sera per Ag. For fourteen of the Ags the monoplex and multiplex assay show similar results. For some of the Ags (GLURP, LSA1-41, LSA1-J, Pf GLURP R2, SALIV2 and PvDBP) the multiplex assay gives a higher signal than the monoplex assay.

Antigens	Correlation coefficient (R^2)	p- value
LSA1-J	0.8956	<0.001
GLURP	0.9231	<0.001
LSA1-41	0.7717	0.01187
PvDBP	0.9546	<0.001
PfMSP1-19	0.973	<0.001
Pf GLURP R2	0.9643	<0.001
STARP-R	0.9634	0.001275
Pf13	0.9948	<0.001
LSA3-RE	0.9942	<0.001
SALSA2	0.9873	<0.001
GLURP P3	0.994	<0.001
SALIV2	0.8048	<0.001
CSP	0.8872	0.0020777
PmCSP	0.998	<0.001
PvVK210 CSP	0.9568	<0.001
PvMSP1-19	0.9888	<0.001
SR11.1	0.995	<0.001
Pv like CSP	0.9913	<0.001
SALSA1	0.9405	<0.001

Table 9: Correlation coefficient (R2) and the p-value generated in the Spearman Rank test for comparing the monoplex and multiplex from the individual sera tested per Ag.

4.4 Influence of the amount of beads and detecting Ab per Ag per well

The difference between the amounts of beads/Ag/well is tested for 1000 and 2000 beads/Ag/well. Figure 20 shows results (Δ MFI) obtained from the exposed pooled sera (1:100 dilution) which are incubated with the 20 tested Ag-coated beads. The results between the 1000 and 2000 beads/Ag/well are almost identical for most of the Ags. According to the graph in Figure 20 the ΔMFI values for 1000 beads/Ag/well seems to be a little bit higher than for 2000 beads/Ag/well.

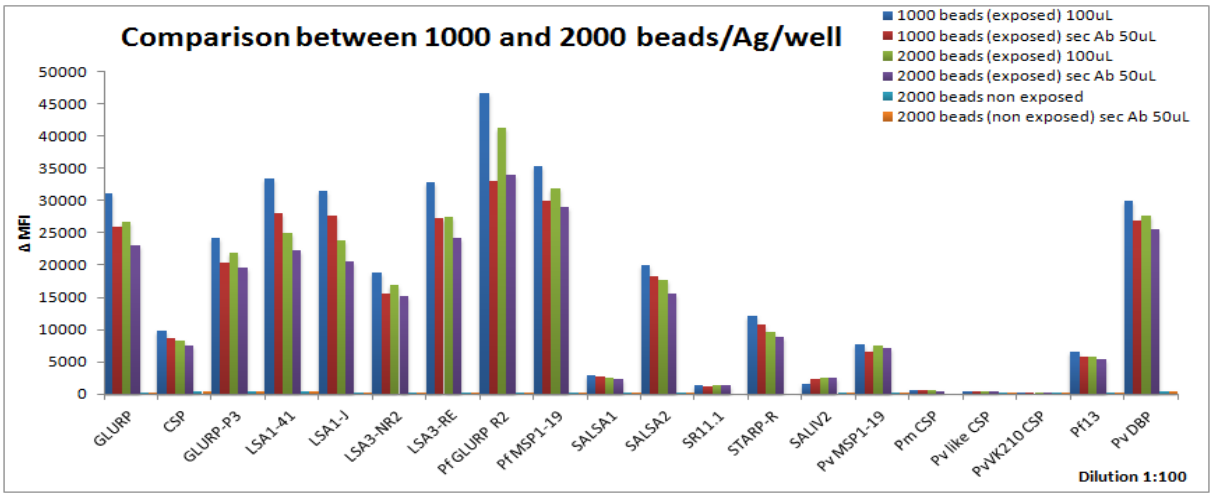


Figure 20: The positive sera pool and the unexposed sera are tested on the multiplex assay (dilution 1:100) for 1000 beads/Ag/well and 2000 beads/Ag/well and for 100µL and 50µL of detecting Ab. The blue and red bars show results of 1000 beads/Ag/well and the green and purple bars show results of 2000 beads/Ag/well.

Table 10 shows the comparison (correlation coefficients and p-values calculated with the Spearman Rank correlation test) of the use of 1000 and 2000 beads/well for each Ag tested on the pooled positive control sera (1:100). The results are shown for 100µL and 50µL of detection Ab. The correlation coefficient (R^2) indicates for both 100µL and 50µL that there is a good correlation between the use of 1000 and 2000 beads/Ag/well. There is chosen to use 100µL of detection Ab, because the results in Figure 20 and the R^2 values shown in Table 10 are a bit better then the use of 50µL of detection Ab.

Antigens	Correlation coefficient (R^2) 100µL detection Ab	p- value	Correlation coefficient (R^2) 50µL detection Ab	p- value
GLURP	0.9940	4.96E-05	0.9960	4.96E-05
CSP	0.9995	5.51E-06	0.9910	5.51E-06
GLURP-P3	0.9490	5.51E-06	0.9834	5.51E-06
LSA1-41	0.9696	0.00035	0.9869	0.00035
LSA1-J	0.9765	4.96E-05	0.9105	0.00308
LSA3-NR2	0.9965	5.51E-06	0.9796	5.51E-06
LSA3-RE	0.9921	4.96E-05	0.9947	4.96E-05
Pf GLURP R2	0.9953	5.51E-06	0.9510	0.00035
Pf MSP1-19	0.9953	0.00203	0.9901	0.00131
SALSA1	0.9966	5.51E-06	0.9981	5.51E-06
SALSA2	0.9995	5.51E-06	0.9982	5.51E-06
SR11.1	0.9791	5.51E-06	0.9841	5.51E-06
STARP-R	0.9980	5.51E-06	0.9948	5.51E-06
SALIV2	0.9203	4.96E-05	0.9657	5.51E-06
PvMSP1-19	0.9973	5.51E-06	0.9742	0.00083
PmCSP	0.9959	5.51E-06	0.9940	0.00075
Pv like CSP	0.9963	4.96E-05	0.9924	5.51E-06
PvVK210 CSP	0.9862	5.51E-06	0.9567	4.96E-05
Pf13	0.9998	5.51E-06	0.9937	5.51E-06
Pv DBP	0.9977	5.51E-06	0.9909	5.51E-06

Table 10: The correlation coefficients and p-values of the 1000 beads/Ag/well versus 2000 beads/Ag/well calculated for 100µL of detection Ab and 50µL detection Ab.

The 3 dilutions (1:100, 1:400 and 1:1600) selected for the bead-based immunoassay are also tested on 1000 and 2000 beads/well for each Ag separately. The three dilutions are tested in triplicate per 1000 beads/well and per 2000 beads/well. There is no significant difference between the use of 1000 and 2000 beads/Ag/well (Kruskal-Wallis test, $p>0.05$ for each Ag). Figure 21 shows a clear separation of each dilution. A calculation of the standard deviation is done. For Ags with a high ΔMFI value the standard deviations are low, which indicates a minimal variation in ΔMFI value for each dilution. Each Ag shows that the ΔMFI value for 1000 bead/well are slightly higher than for 2000 beads/well. Therefore, according to all results we will continue the bead-based immunoassay with an amount of 1000 beads/well.

Antigens	P-value
GLURP	0.145
CSP	0.354
GLURP-P3	0.354
LSA1-41	0.102
LSA1-J	0.085
LSA3-NR2	0.354
LSA3-RE	0.102
Pf GLURP R2	0.402
Pf MSP1-19	0.171
SALSA1	0.508
SALSA2	0.354
SR11.1	0.691
STARP-R	0.354
SALIV2	0.508
PvMSP1-19	0.895
PmCSP	0.354
Pv like CSP	0.508
PvVK210 CSP	0.825
Pf13	0.354
Pv DBP	0.354

Table 11: The p-values based on the Kruskal-Wallis test calculated for each Ag, to see if there is a difference between the use of 1000 and 2000 beads/Ag/well.

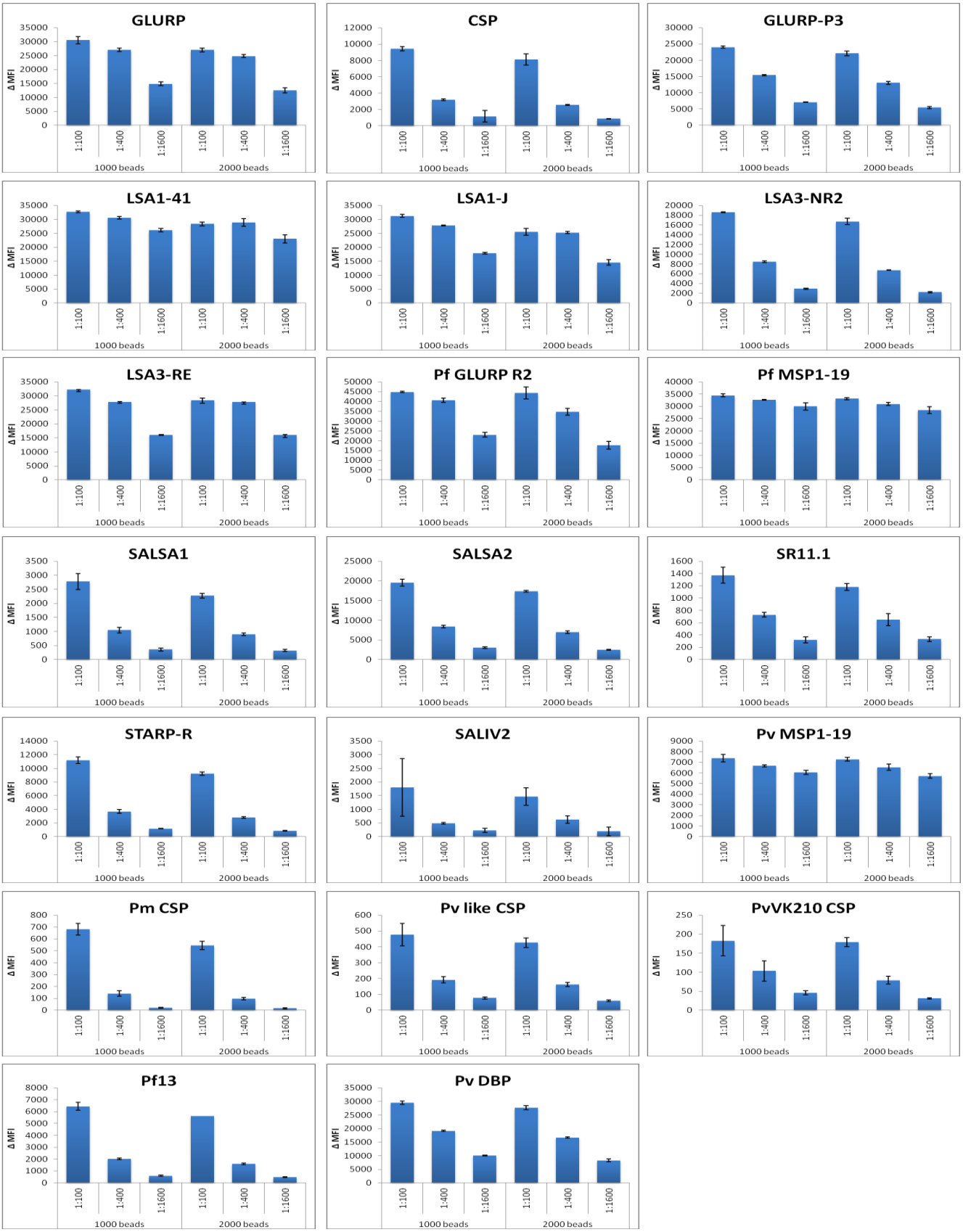


Figure 21: Comparison between use of 1000 and 2000 beads/ well calculated for each Ag, per dilution. The bars show a clear separation of each dilution. The ΔMFI for 1000 beads/well are a little bit higher than for 2000 beads/well. The standard deviation error bars (3 assays) confirm that there is a minimal variation in ΔMFI value around the mean per dilution.

4.5 *Defining of the serum dilutions in multiplex assays for a high positive (100%) and medium positive (targeted at 50%) controls*

The pool of sera was tested in the multiplex assay in triplicate per dilution. For each Ag a high positive (100%) and medium positive (targeted at 50%) of the positive control pooled sera is chosen by making a titration curve as described in Figure 11 (Material and Methods) for each Ag (Table 12 and Figure 22).

Observing the serum dilutions, the highest ΔMFI values for most of the Ags were seen in the lowest 1:100 dilution (Figure 22 and Figure 23) within multiplex testing of all Ags. For some of the Ags the 1:100 dilution was in the plateau phase. For these Ags, the 1:400 dilution is chosen as the highest MFI. Furthermore the 1:400 dilution is used as the low (50%) positive concentration for some of the Ags and for the others the 1:1600 dilution is used (Table 12). Based on these results, the pool of sera was prepared at dilutions 1:100, 1:400 and 1:1600 to be used as positive controls in all plates.

Antigens	1:100 ΔMFI (mean)	1:400 ΔMFI (mean)	1:1600 ΔMFI (mean)
GLURP	30525	27099	14843
CSP	9424	3197	1154
GLURP-P3	24022	15446	7078
LSA1-41	32771	30594	26114
LSA1-J	31273	27837	17875
LSA3-NR2	18633	8445	2921
LSA3-RE	32196	27807	16076
Pf GLURP R2	44868	40664	22941
Pf MSP1-19	34492	32677	30023
SALSA1	2781	1051	359
SALSA2	19556	8407	3061
SR11.1	1373	729	322
STARP-R	11195	3676	1161
SALIV2	1806	491	222
Pv MSP1-19	7389	6671	6062
Pm CSP	681	141	21
Pv like CSP	478	192	78
PvVK210 CSP	183	104	46
Pf13	6446	2026	598
PvDBP	29538	19183	10067

Table 12: Results (ΔMFI (mean)) obtained per Ag with the multiplex assay on the pooled sera for the three chosen dilutions as strong positive (dark blue) and weak positive (light blue) controls (1:100, 1:400, 1:1600).

These dilutions were assessed (Figure 22 and ANNEX 4) by testing them in multiplex on the same plate in triplicate. The triplicate results are then compared to each other to see if the results are similar (see previous paragraph). This is performed before starting testing of the samples. ANNEX 4 shows the results of each dilution per Ag tested in triplicate and Figure 23 shows the average of the results of each dilution per Ag tested. Based on the results in Figure 22, Figure 23 and ANNEX 4 (table and graphs) the three dilutions prepared from the pooled sera are consistent and useful for the final multiplex assay.

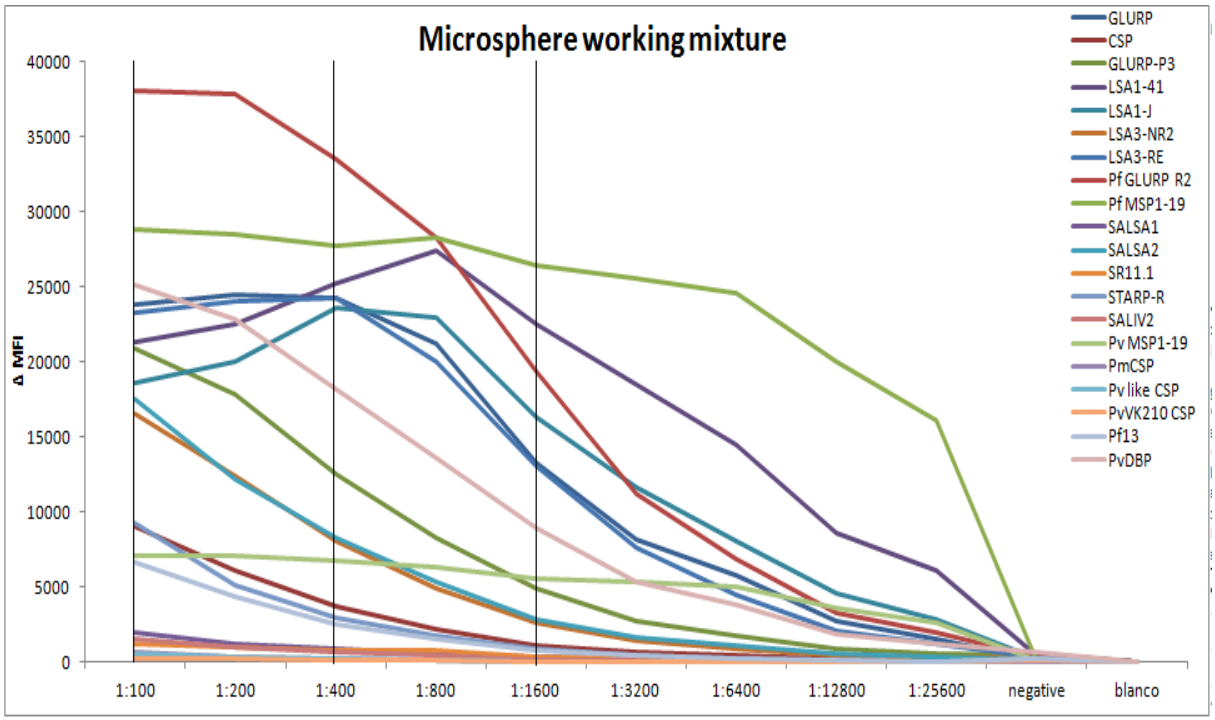


Figure 22: Multiplex assay on a dilution series of the positive control pooled sera for determining the optimal serum dilution. The vertical lines indicate the dilutions chosen to be used in the final multiplex assay.

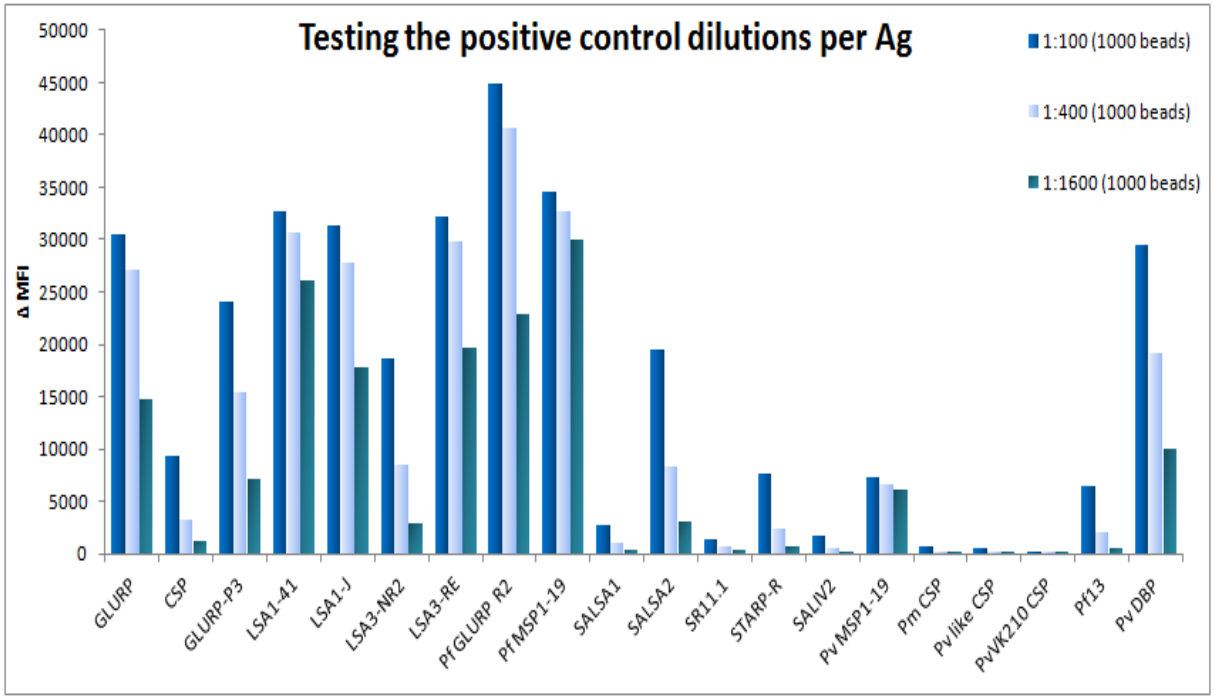


Figure 23: Testing of the positive control by using 1000 beads per Ag per well. This graph shows the results obtained for the exposed pooled sera for the three chosen dilutions as a strong positive and weak positive control (1:100, 1:400 and 1:1600). The graph shows that the dilutions are a good control for all the peptides and proteins. This even shows that the pooled sera work for every dilution.

4.6 Comparison ELISA and Multiplex assay results

Furthermore, the results of the percent positivity (%) from the pooled positive control sera are compared to those obtained with the ELISA. The comparison is done on the 1:400 dilution, because a sera dilution of 1:400 was used for the ELISA (Figure 23). The results obtained with the multiplex assay are lower than the results from the ELISA. This can be due to a lower amount of proteins that bind to the beads in the multiplex assay. There is a similarity in the Ags that give a high signal and a low signal compared to the recombinant Ag Pf GLURP R2 in the ELISA and the multiplex assay. However, some Ags (SALSA1, SR11.1 and Pv MSP1-19) react differently in the multiplex assay (Figure 23).

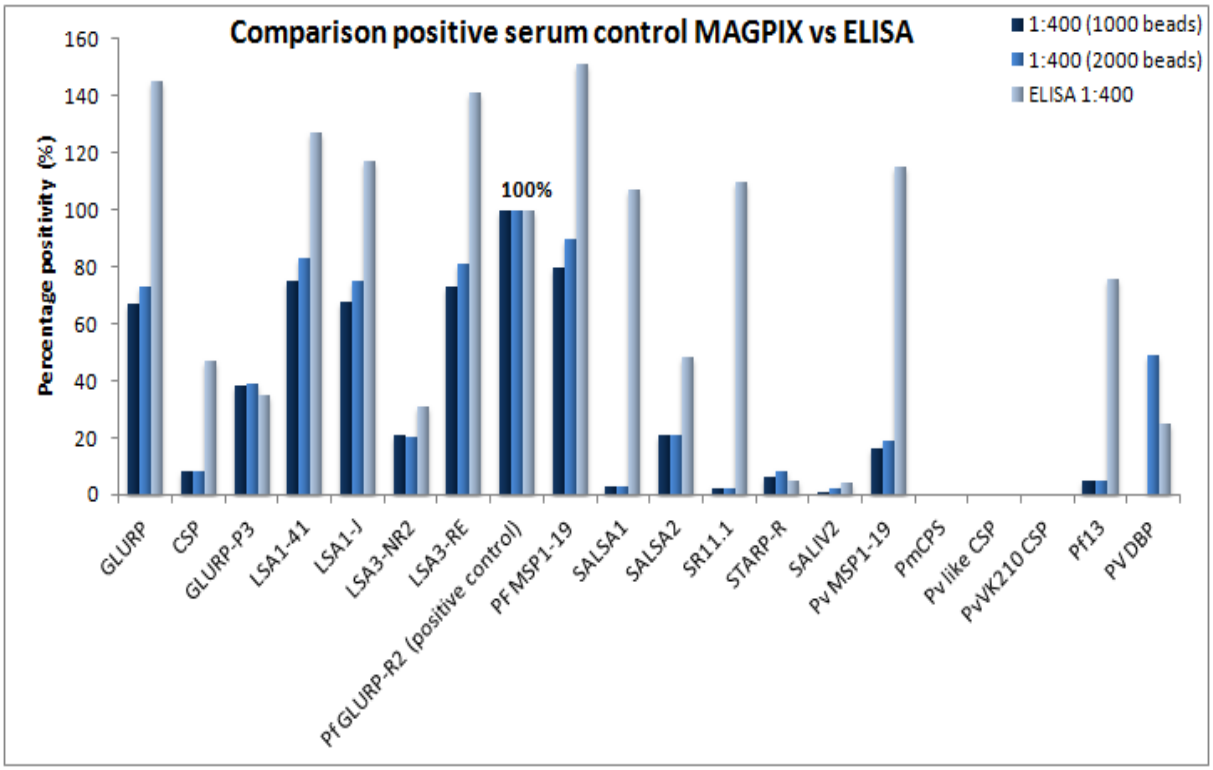


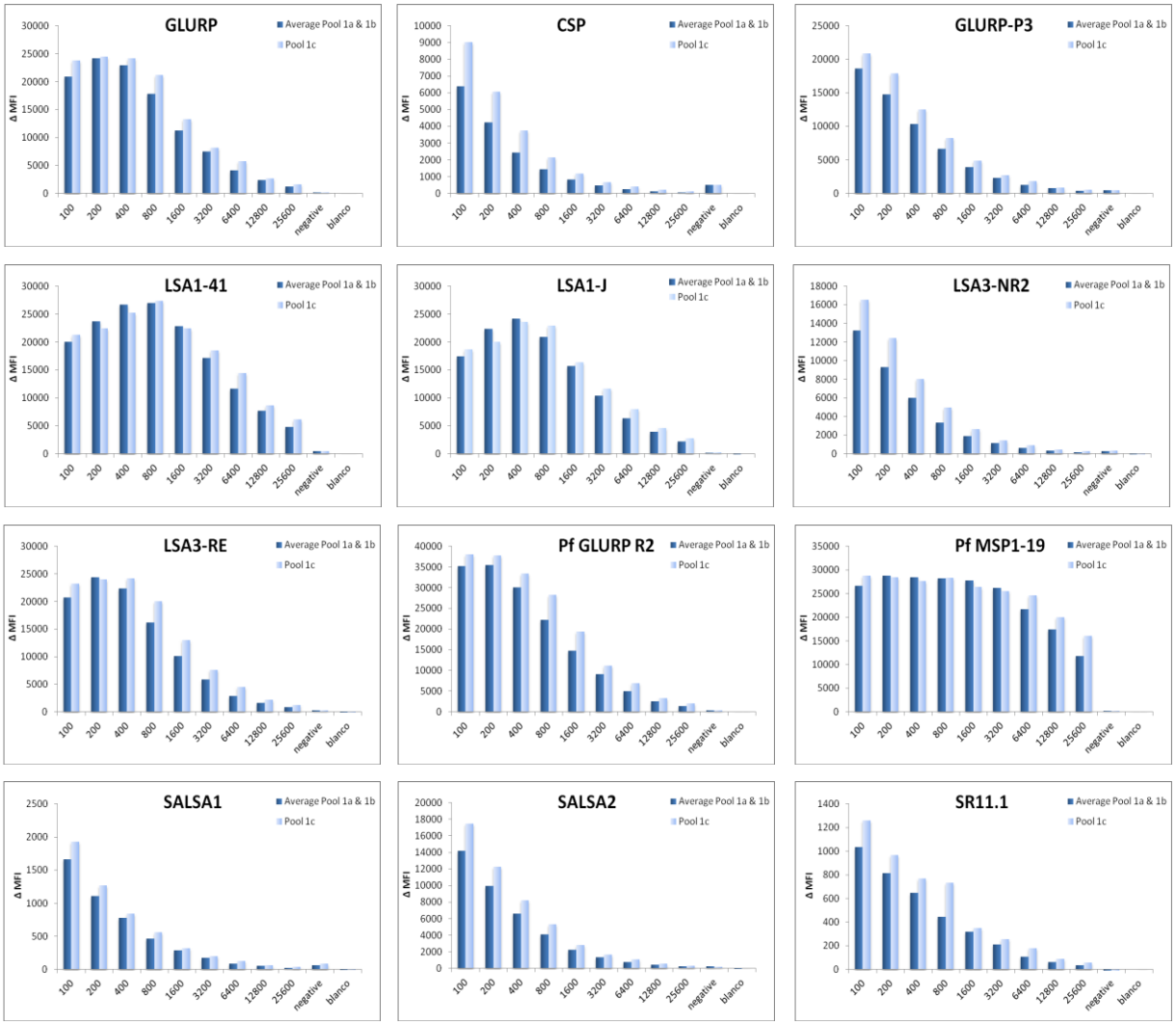
Figure 24: Percentage positivity (%) form the pooled positive control sera obtained with the multiplex assay, compared with the percentage obtained by the ELISA. The recombinant Ag Pf GLURP R2 was used as reference (100%) for the multiplex assay. The bar graph shows higher results for the ELISA than for the multiplex assay, for most of the Ags (GLURP, CSP, LSA1-41, LSA1-J, LSA3-RE, Pf GLURP R2, SALSA1, SALSA2, SR11.1, Pv MSP1-19, Pf13 and Pv DBP).

4.7 Interassay reproducibility of the multiplex assay

Multiplex assay (20 Ags) was performed on two plates (plate one (pool 1a & 1b) and plate two (pool 1c)) for the pooled sera dilution series (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600) on two different days to see if there is a similarity in the repeatability between the two plates. Figure 23 shows the graphs of each Ag separately, which make clear that there is no big difference between the two different plates for GLURP, GLURP-P3, LSA1-41, LSA1-J, LSA3-RE, Pf GLURP R2, Pf MSP1-19, SALSA1, Pv MSP1-19, PvVK210 CSP and Pv DBP). For the other Ags the graphs show a difference between the two plates. Table 13 shows the correlation coefficient (R2) and the p-values based on the Spearman Rank correlation test for each Ag obtained with the control positive pooled sera based on the ΔMFI values. The high R2 results and the $p<0.001$ (for all Ags) indicate that there is a good correlation between the two plates.

Antigens	Correlation coefficient (R^2)	P-values
LSA1-J	0.9824	<0.001
GLURP	0.9905	<0.001
LSA1-41	0.9860	<0.001
PvDBP	0.9946	<0.001
PfMSP1-19	0.9724	<0.001
Pf GLURP R2	0.9908	0.010769
STARP-R	0.9991	<0.001
Pf13	0.9977	<0.001
LSA3-RE	0.9846	<0.001
GLURP P3	0.9972	<0.001
SALSA2	0.9997	<0.001
GLURP-P3	0.9972	<0.001
SALIV2	0.9851	<0.001
CSP	0.9986	<0.001
PmCSP	0.9891	<0.001
PvVK210 CSP	0.9748	<0.001
PvMSP1-19	0.9875	<0.001
SR11.1	0.9806	<0.001
Pv like CSP	0.9635	<0.001
SALSA1	0.9990	<0.001

Table 13: Interassay reproducibility of the multiplex assay. This table shows the correlation coefficient (R^2) of each Ag. The pooled sera is tested on two different plates. The obtained results are compared with each other per Ag. P-values are based on the Spearman Rank Correlation test. The table shows that all the R^2-values are high, which indicate that there is no significant difference between the two plates.



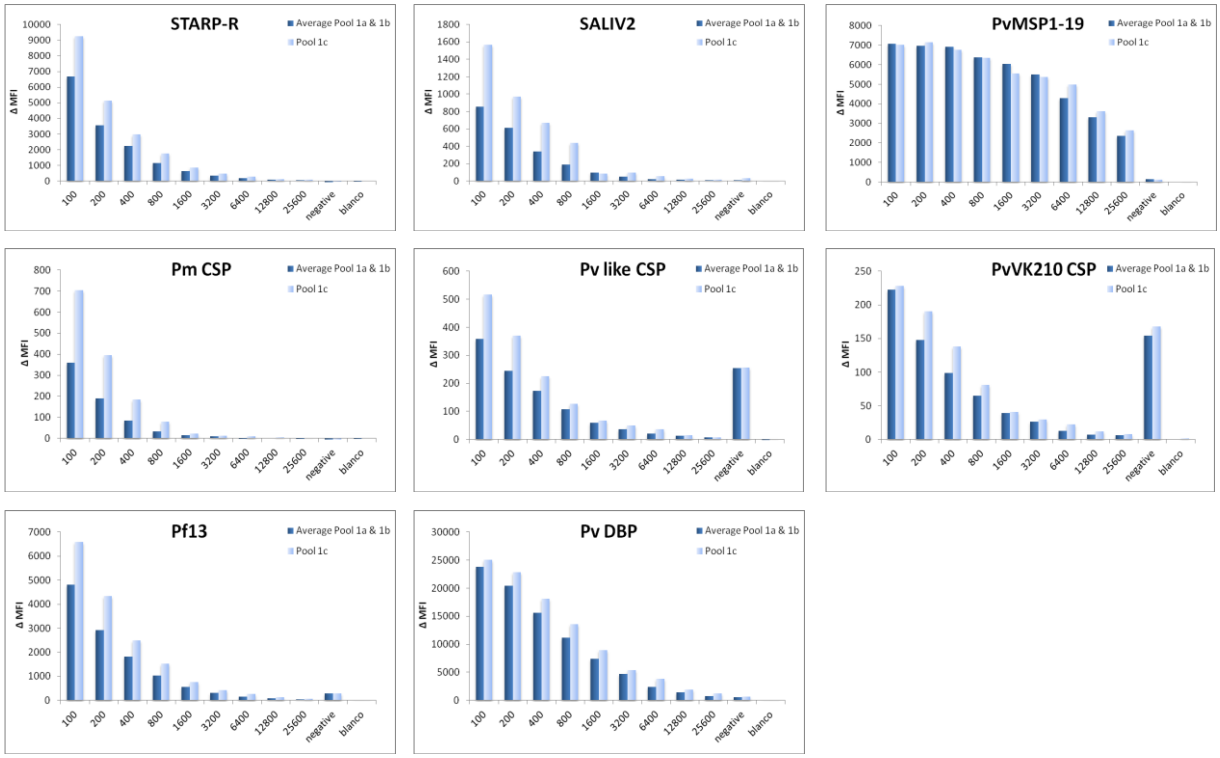


Figure 25: Interassay reproducibility of the multiplex assay shown per Ag. In these graphs the pooled sera is tested on the multiplex assay on two different plates. The obtained results are compared with each other per Ag. The graphs show that there is a similarity between the two different plates per Ag, which indicates that there is no difference between the two assays.

4.8 Quality control of the high positive control and calculation of the cut-of values for sero-positivity per Ag

Of the 2000 collected samples, 1440 samples were screened with the MAGPIX of which 1360 sample results were validated for their positive control used on all Ags. For these Ags Table 14 shows the ΔMFI (mean), the lower and upper limit and the total amount of rejected samples all calculated per Ag.

Antigens	Mean ΔMFI	Lower limit -2SD (ΔMFI_{min})	Upper limit +2SD (ΔMFI_{max})	Total # of rejected samples from the 1440	Total # of samples left from the 1360
GLURP	26090	20697	31482	113	1327
CSP	9567	7279	11754	115	1325
GLURP-P3	21817	16222	27413	100	1340
LSA1-41	25902	21268	30536	110	1330
LSA1-J	24484	17425	31543	104	1336
LSA3-NR2	17840	13229	22451	99	1341
LSA3-RE	21170	18399	23941	126	1314
Pf GLURP R2	26536	19623	33448	117	1323
Pf MSP1-19	35173	24844	45501	114	1326
SALSA1	3369	2311	4428	112	1328
SALSA2	19291	14854	23727	112	1328
SR11.1	1393	981	1806	99	1341
STARP-R	12083	9321	14844	107	1333
SALIV2	1367	145	2589	92	1348
PvMSP1-19	6098	3637	8559	107	1333
PmCSP	668	453	884	98	1342
Pv like CSP	520	374	666	91	1349
PvVK210 CSP	159	40	278	98	1329
Pf13	6319	4529	8109	111	1329
Pv DBP	27367	21411	33324	97	1343

Table 14: Means of ΔMFI of the high positive controls, with lower and upper limit, used for validation of the assays per Ag. Total number of assays that were rejected (fall out of the indicated limits) per Ag.

4.9 Proportion of sero-prevalence and specific Ab levels for the used serological markers

The proportion of sero-positivity and the specific Ab responses directed to the used serological markers are presented for all screened blood samples (Figure 26).

In order to be able to compare the Ab responses against the different Ags, in here the raw ΔMFI data are shown. LSA3-RE shows the highest Ab response, with a ΔMFI mean of 6028 (95% CI = (6028 – 8406)), followed by Pf MSP1-19 with a ΔMFI mean of 4647 (95% CI = (4647 – 6087)), and Pf GLURP R2 with a ΔMFI mean of 4449 (95% CI = (4449 – 6933)). Moderate Ab response levels are shown for GLURP, CSP, GLURP-P3, LSA1-41, LSA3-NR2, SALSA1, SALSA2, Pm CSP and Pv DBP with ΔMFI mean values ranging from 614 (95% CI = (614 – 1502)) to 2652 (95% CI = (2652 – 4573)). In contrast the other Ags were associated with lower levels in Ab response.

The proportion of sero-positivity was the highest for the LSA3-RE (54.3%), CSP (52.7%) and Pf MSP1-19 (52.1%) peptides. A moderate sero-prevalence was found in GLURP, LSA1-41, Pf GLURP R2, SALSA2, STARP-R and PvVK210 CSP. For all the other Ags a very low proportion of sero-positivity was observed.

These results show that pre-erythrocytic LSA3-RE, CSP, GLURP, LSA1-41, SALSA2, STARP-R and PvVK210 CSP peptides and Pf MSP1-19 and Pf GLURP R2 recombinant protein appear to be most antigenic, giving the highest proportion of sero-positivity. These Ags could be potential serological markers of malaria transmission.

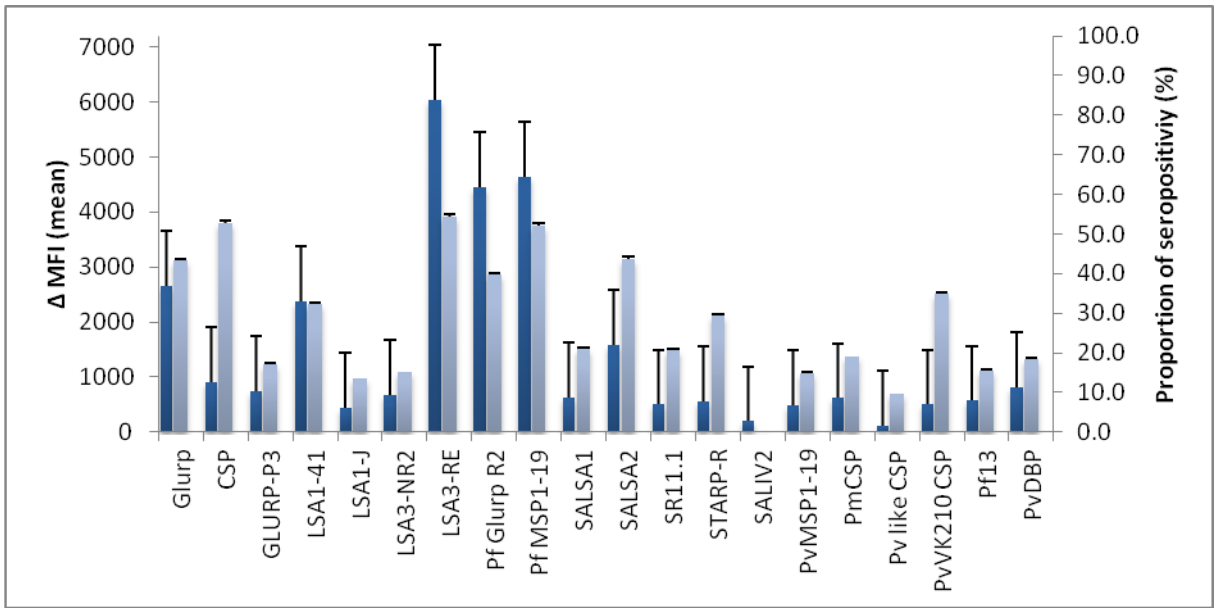


Figure 26: Proportion of sero-prevalence and specific Ab levels for the used serological markers. The dark blue bars indicate the Ab levels (ΔMFI (mean)= MFI_{Ab} – MFI_{BSA}) and the light blue bars the proportion of sero-positivity (%). The error bars are the upper limits of the 95% confidence intervals.

4.10 Percent positivity in serology according to the PCR results

The percent positivity for each Ag is compared between *Plasmodium* PCR positives and negative samples. As seen in Figure 27 a clear separation between the Pf-negative and Pf-positive PCR results is seen ($p<0.05$) for Ags LSA3-RE, Pf GLURP R2, Pf MSP1-19, GLURP, LSA1-41, GLURP-P3, SALSA2 and SR11.1.

The percent positivity in relation to *P. vivax* PCR results is shown in Figure 28. Only for Ags Pf MSP1-19, Pv DBP, Pv MSP1-19 and PvVK210 CSP a distinction between the two groups (Pv-positive and Pv-negative) was observed ($P<0.05$). All the other ags have a p-value >0.05, which indicates that there is no difference between the two groups.

For *P. malariae* PCR results the percent positivity is presented in Figure 29. A significant difference ($p<0.05$) between the two groups (Pm-positive and Pm-negative) is seen for Pm CSP, Pf GLURP R2 and CSP (Pf). All other Ags show no difference between the two groups.

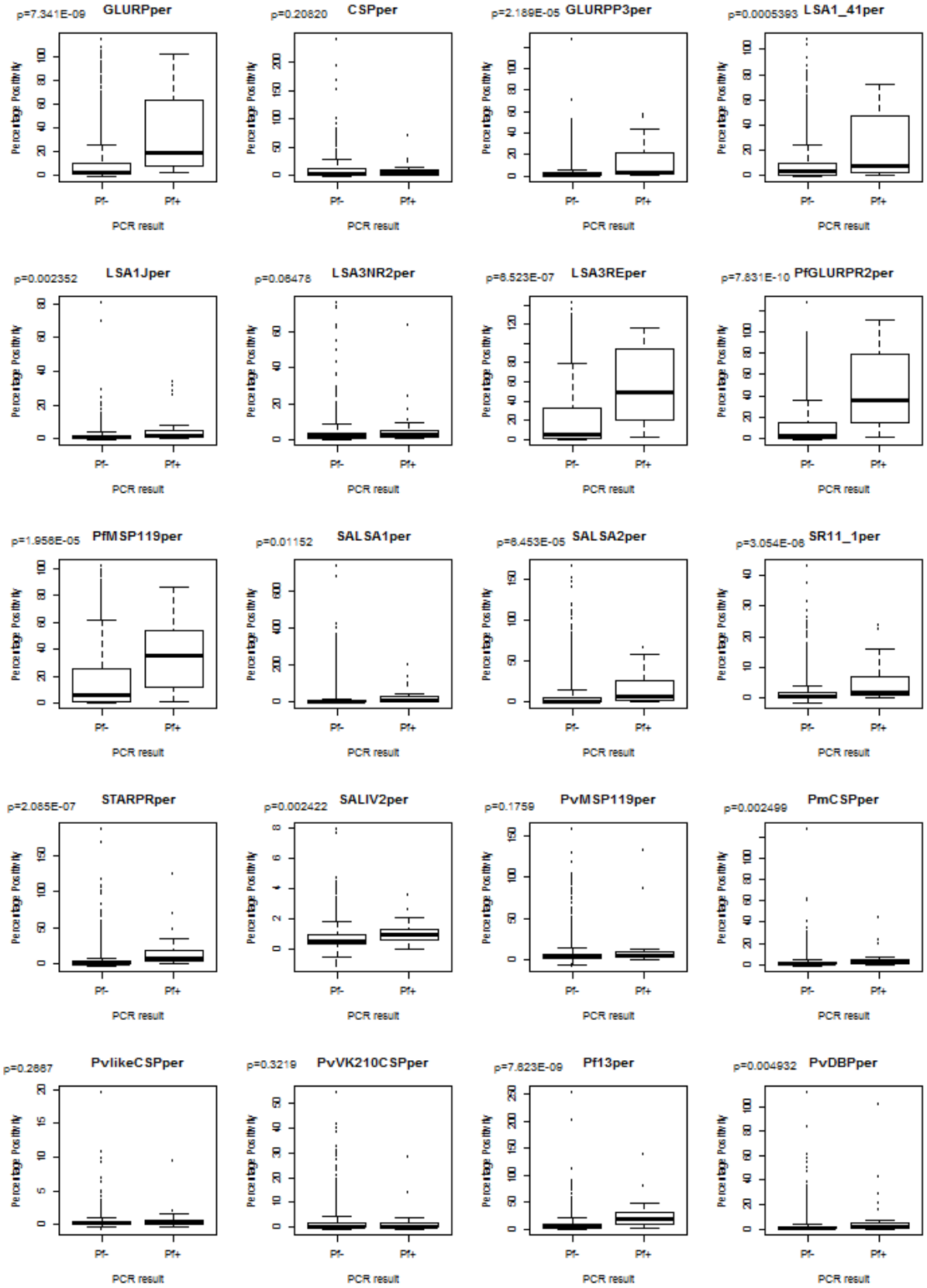


Figure 27: Percent positivity in serology according to the PCR results. The lines in the box plot represents 75th percentile, median and 25th percentile of the individual percent positivity. Whiskers represent the maximal and minimal outlier limits. Dots represent extreme outliers. Differences between groups were tested using the Mann-Whitney U test.

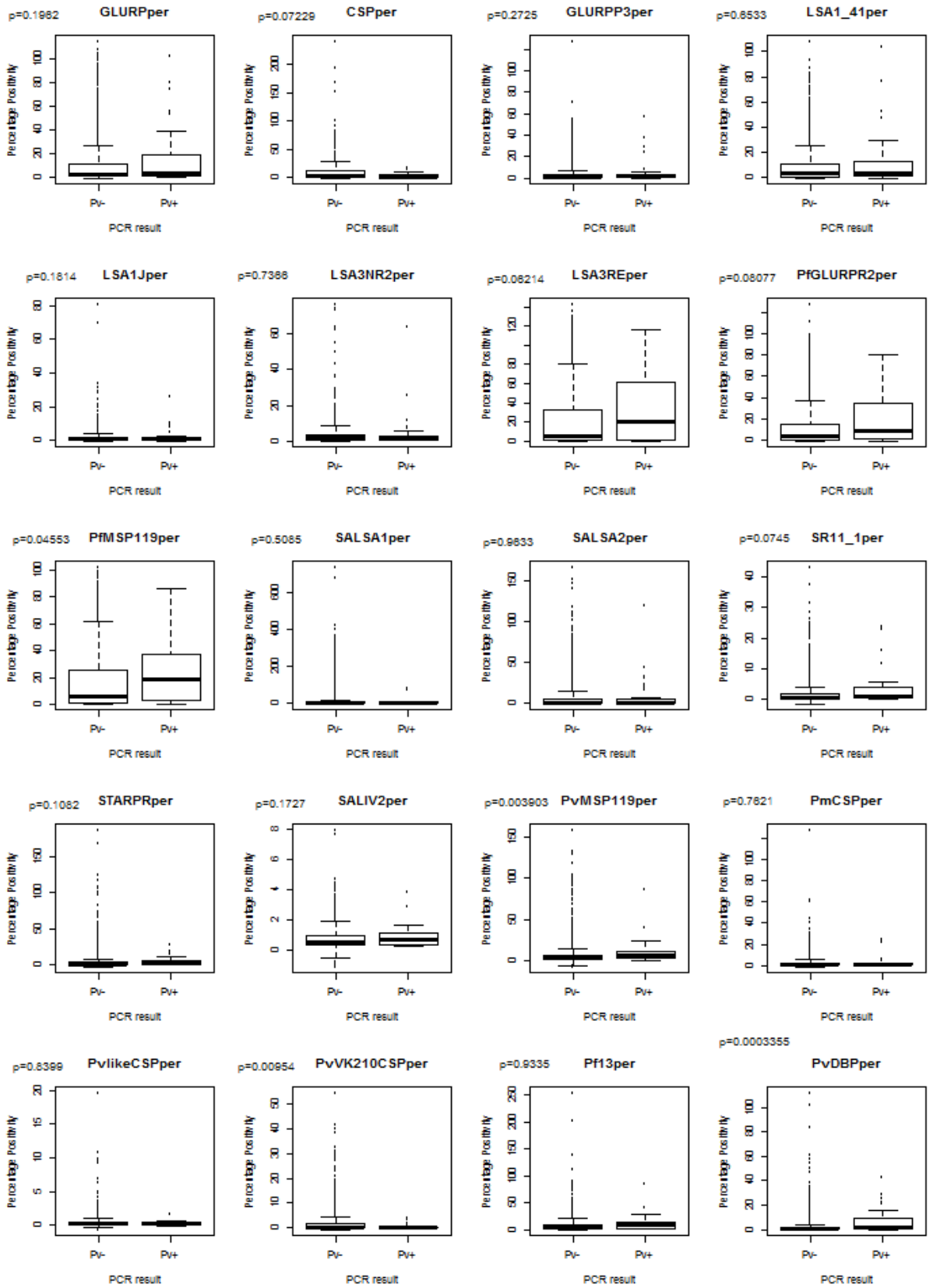


Figure 28: Percent positivity in serology according to the PCR results. The lines in the box plot represents 75th percentile, median and 25th percentile of the individual percent positivity. Whiskers represent the maximal and minimal outlier limits. Dots represent extreme outliers. Differences between groups were tested using the Mann-Whitney U test.

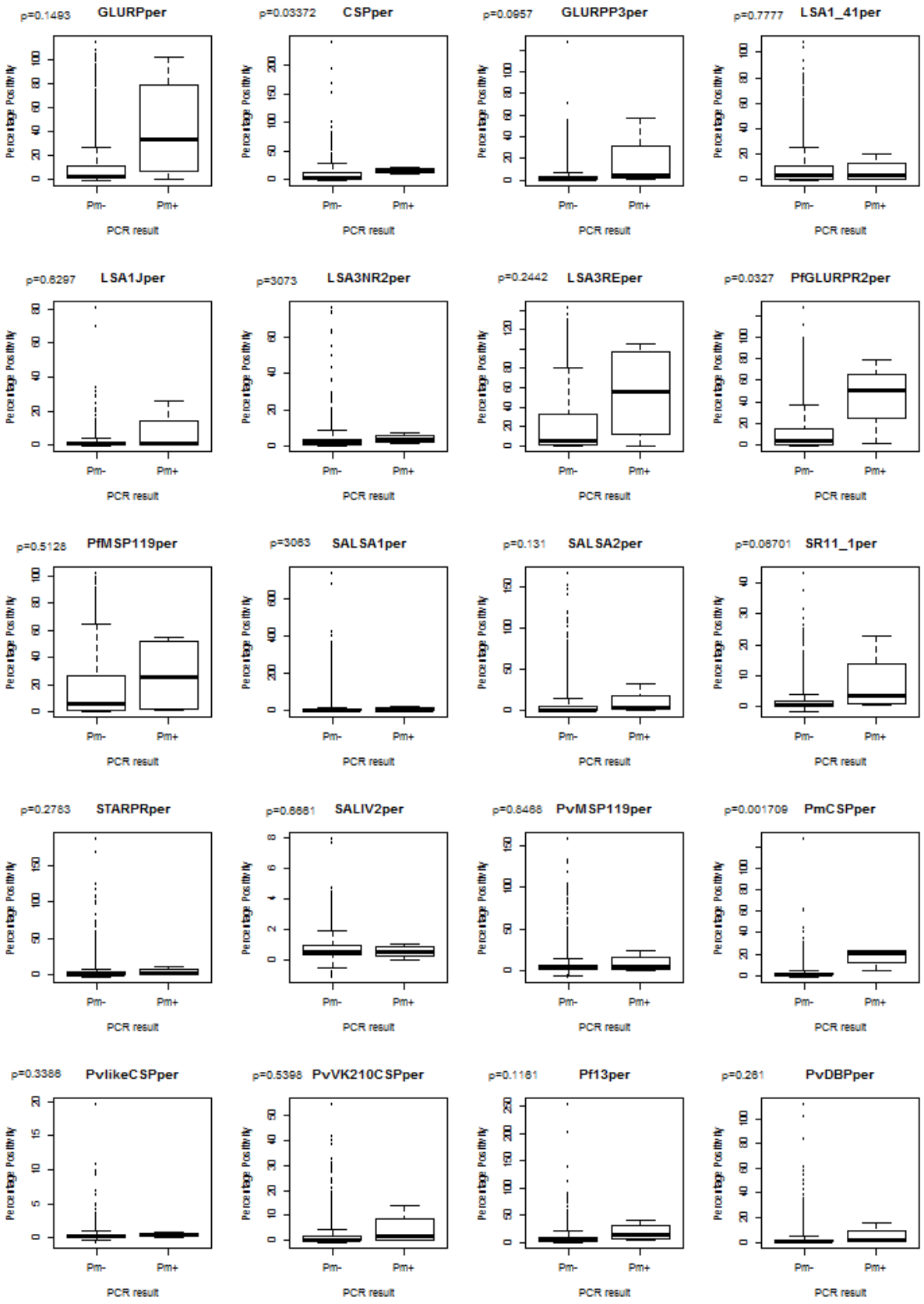


Figure 29: Percent positivity in serology according to the PCR results. The lines in the box plot represents 75th percentile, median and 25th percentile of the individual percent positivity. Whiskers represent the maximal and minimal outlier limits. Dots represent extreme outliers. Differences between groups were tested using the Mann-Whitney U test.

4.11 Number of Ags recognized by PCR positive and negative individuals

1274 samples (with validated results for all Ags) are used for determining the percentage of people responding to zero, one of more Ags.

The bar graphs in Figure 30 gives the number of collected samples responding positive to zero, one or more Ags for *P. falciparum* and *P. vivax*. For *P. falciparum* and *P. vivax* most of the individuals respond to >1 Ag (respectively 158 (12.4%) and 329 (25.8%)). The results in Table 15 show that most individuals respond positive to the following Pf Ags: LSA-RE, CSP, Pf MSP1-19, GLURP, SALSA2 and Pf GLURP R2. This is possible due to the half life of the Ags or to the produced Ab-levels.

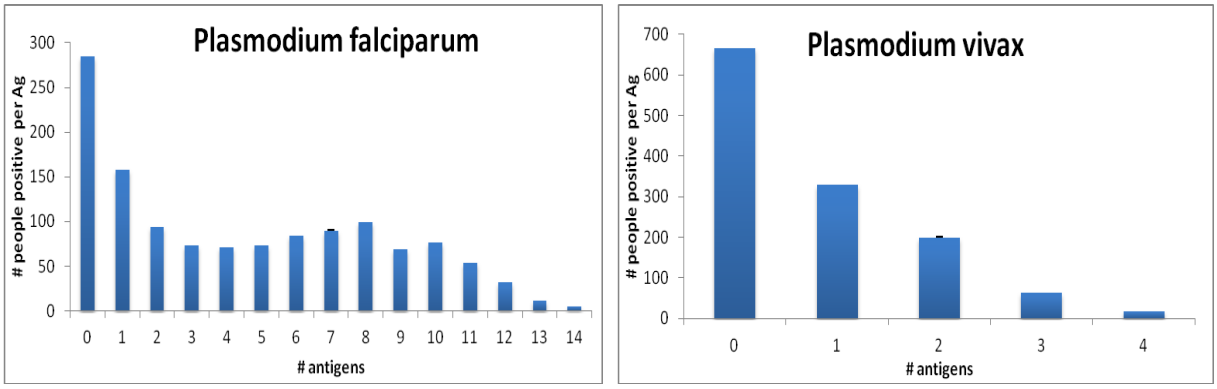


Figure 30: Number of collected samples (1274) responding positive to zero, one or more antigens of *P. falciparum* and *P. vivax*.

Pf # Ags	Glurp	CSP	GLURP-P3	LSA1-41	LSA1-J	LSA3-NR2	LSA3-RE	Pf Glurp R2	Pf MSP1-19	SALSA1	SALSA2	SR11.1	STARP-R	SALIV2	Pf13	Total
1	13	30	0	4	1	14	16	7	43	2	15	4	6	0	3	158
2	14	39	2	19	6	13	25	1	30	3	25	1	8	0	2	94
3	14	44	3	14	8	11	33	9	37	7	24	2	9	0	4	73
4	14	42	6	19	7	9	48	14	41	11	41	5	22	0	5	71
5	42	54	6	35	8	8	60	31	47	14	35	6	11	0	8	73
6	51	67	8	31	11	10	78	44	64	18	52	17	35	1	17	84
7	72	68	13	39	14	16	83	71	75	23	62	26	44	0	24	90
8	89	88	33	56	17	11	98	92	86	35	64	41	61	0	21	99
9	67	65	31	43	11	17	69	64	60	35	59	34	41	0	25	69
10	73	71	36	58	34	30	76	72	74	45	73	41	54	1	22	76
11	54	51	40	44	23	25	54	54	53	38	53	36	41	0	28	54
12	31	32	25	29	17	19	32	32	31	24	31	26	28	0	27	21
13	11	10	10	11	11	10	11	11	11	8	10	11	11	0	7	11
14	5	5	5	5	5	5	5	5	5	5	5	5	5	0	5	5
Total	550	666	218	407	173	198	688	507	657	268	549	255	376	2	198	

Table 15: Number of collected samples responding positive to one or more Ags of *P. falciparum* determined for each Ag separately. Most individuals respond for LSA3-RE, CSP, Pf MSP1-19, GLURP, SALSA2 and Pf GLURP R2.

Pv # Ags	PvMSP1-19	Pv like CSP	PvVK210 CSP	PvDBP	Total
1	43	16	202	68	329
2	88	62	156	94	200
3	46	23	61	59	63
4	17	17	17	17	17
Total	194	118	436	238	

Table 16: Number of collected samples responding positive to one or more Ags of *P. vivax* determined for each Ag separately. Most individuals respond for PvVK210 CSP and PvDBP.

PCR-positive and -negative status was analyzed with SPSS v17 (Graphs <Legacy <dialogs <Boxplot <Simple <variable (# Ags positive for Pf) <category Axis (PCR positives for Pf)) in relation to the amount of Ags that people responded to for Pf and Pv separately. The results for *P. falciparum* in Figure 31 show that the PCR negative individuals (median = 4) respond to a lower number of Ags than the PCR positive individuals (median = 9). The results for *P. vivax* also show that the PCR negatives (median = 0; 75th percentile = 1) respond to a lower amount of Ags than the PCR positives (median = 1). In this box plot seven outliers are seen, which stand for 7 individuals that are tested negative for the PCR, but of which three responded positive for three different AGs and four responded positive for four different Ags.

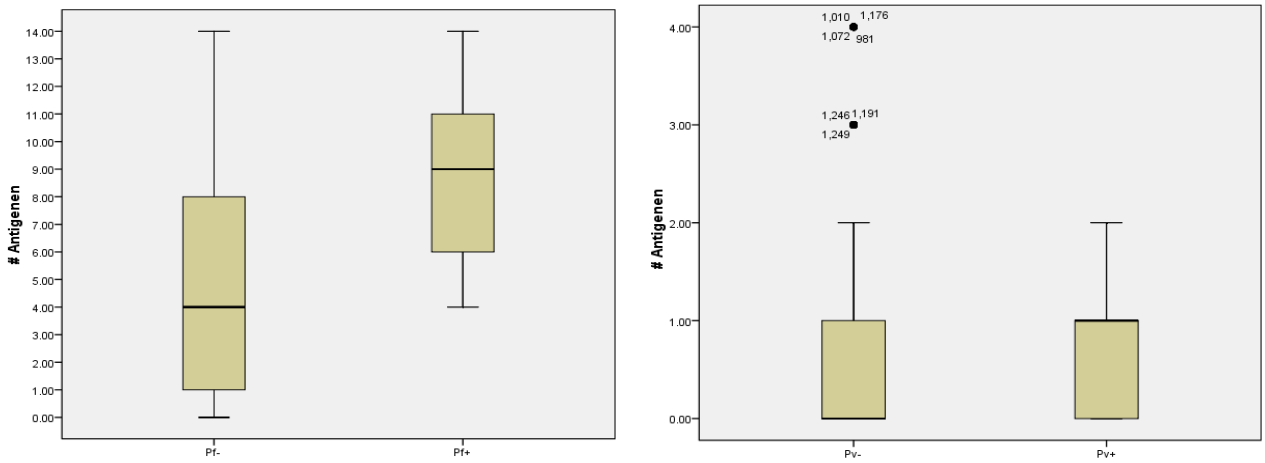


Figure 31: PCR-positive and -negative status in relation to the number of Ags of Pf and Pv that individuals respond to. Fifteen Ags are used for *P falciparum*, of which people responded against a minimum of one and maximum of fourteen Ags. Four Ags are used for *P. vivax* of which people responded against a minimum of one and a maximum of all four Ags.

4.12 Sero-prevalence and number of Ags recognized per age group and gender

The proportion of sero-positivity to at least one Ag and the corresponding average number of detecting specific Abs in individuals were analyzed per age group. This may determine potential serological markers of the exposure to malaria over a prolonged period. Figure 32 shows a clear increase of the sero-positivity to at least one Ag per age group for *P. falciparum*. Furthermore, the results show that the number of Ags recognized is also age-dependent for both *P.falciparum* and *P.vivax* (Kruskal-Wallis test on sero-prositivity p<0.001 for Pv and Pf).

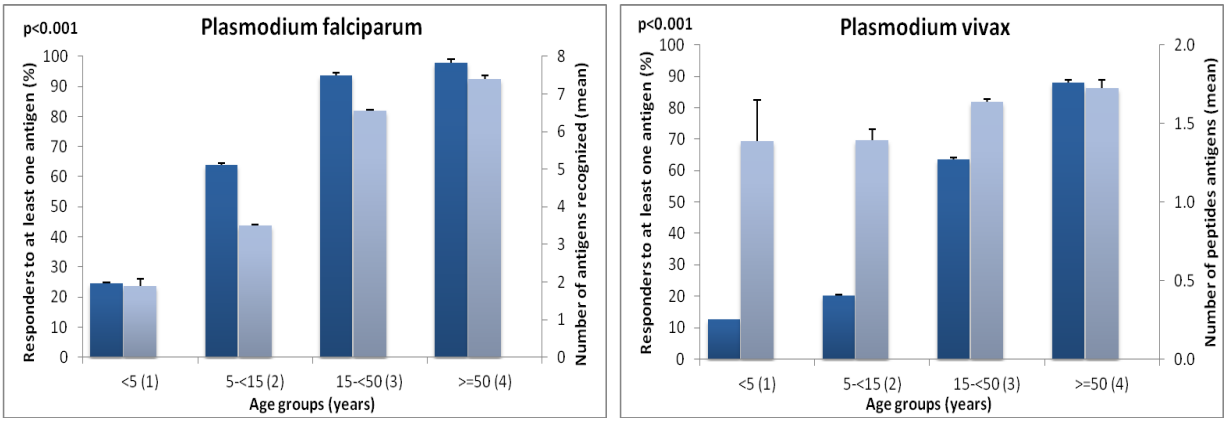


Figure 32: Proportion of sero-positivity to at least one Ag and the corresponding number of Ags tested sero-positive per age group. The dark blue bars indicate the proportion of sero-positivity to at least one Ag and the light blue bars indicate the mean of the corresponding number of Ags recognized. The error bars are the upper limit of the 95% confidence intervals.

All Ags in Figure 33 show a significant increase of sero-positivity per age group (Kruskal-Wallis test P < 0.001) in both graphs. In the graph of *P. falciparum* most persons in all age groups respond highly for LSA3-RE, Pf MSP1-19 and CSP. The two older age groups (15-<50 and ≥50) also show high responses for SALSA2, GLURP and Pf GLURP R2. In the graph of *P. vivax* children <5 respond the same for all 4 Ags, children in the age group 5-<15 respond the highest for Pv DBP, individuals in the age group of 15-<50 and ≥50 respond the highest for PvVK210 CSP.

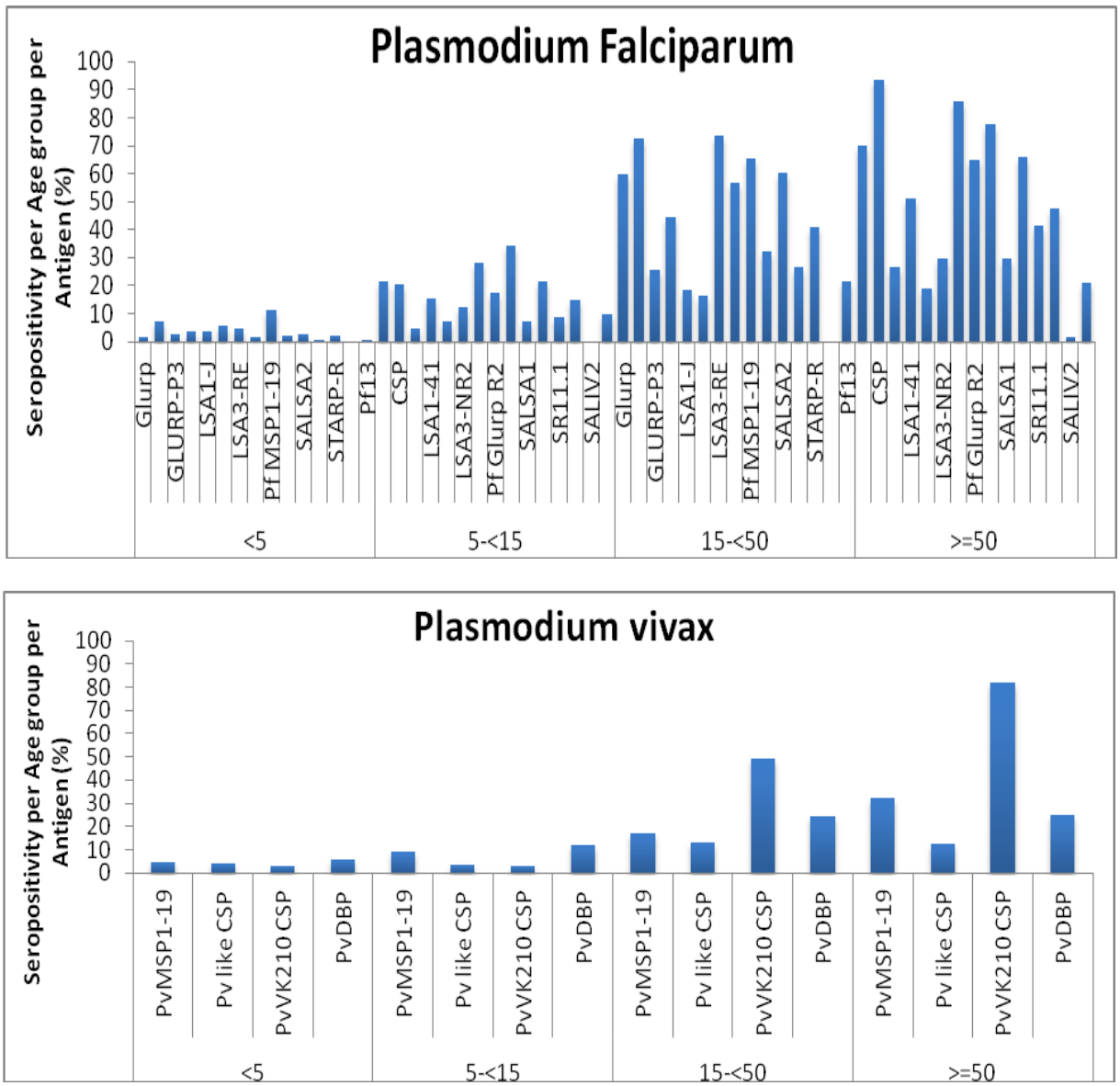


Figure 33: Proportion of sero-positivity per age group and per Ag for *P.falciparum* (upper graph) and *P. vivax* (lower graph).

Furthermore, the proportion of sero-positivity to at least one Ag and the corresponding amount of Ags recognized were analyzed per gender (Figure 34). This is analyzed to see if there is a difference between the rate of malaria infection between men and woman. The results in Figure 34 for both *P. falciparum* and *P. vivax* show that the sero-positivity to at least one Ag and no significant difference is observed between the corresponding number for men and woman (Mann-Whitney U test: p=0.055 and 0.157 for Pf and Pv respectively).

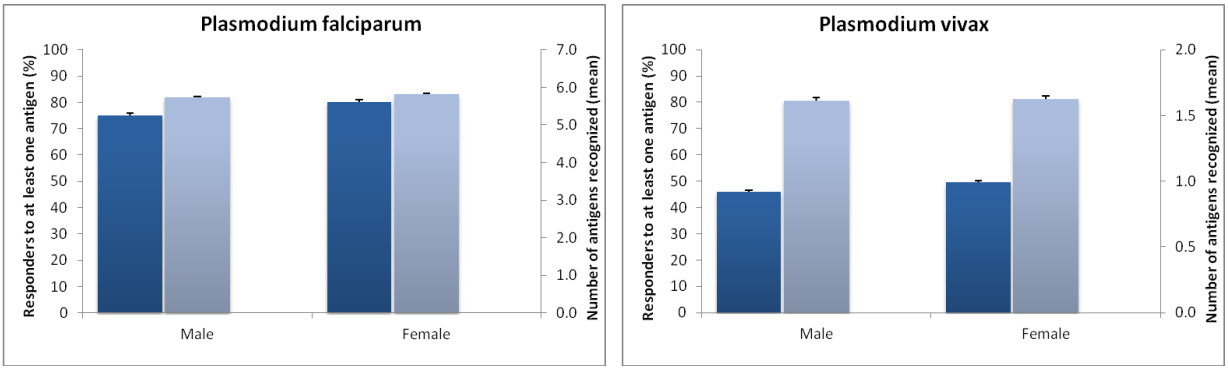
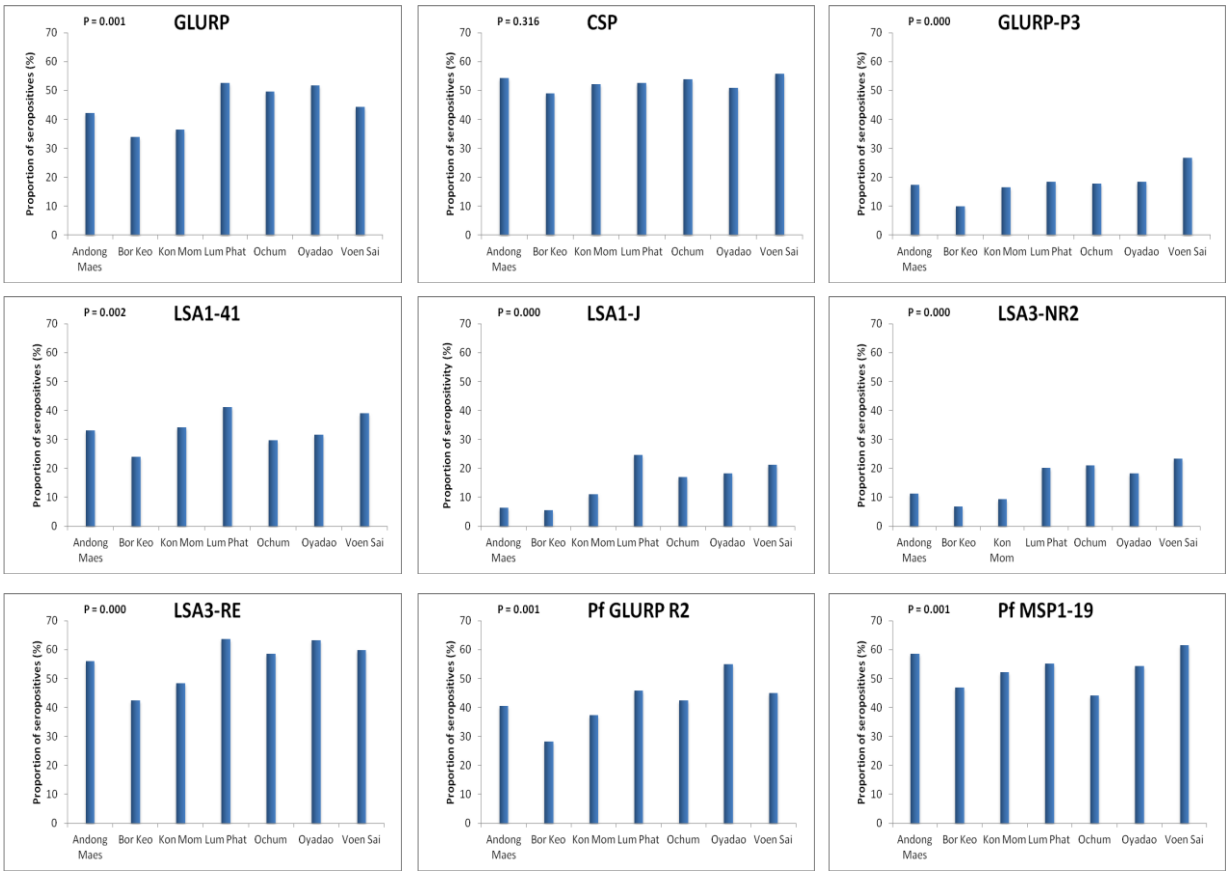


Figure 34: Proportion of sero-positivity to at least one Ag and the corresponding number of Ags tested sero-positive per gender. The dark blue bars indicate the proportion of sero-positivity to at least one Ag and the light blue bars indicate the mean of the corresponding number of Ags recognized. The error bars are the upper limit of the 95% confidence intervals. The p-value (p = 0.055 for Pf and p = 0.157 for Pv) is calculated with the Mann-Whitney U test.

Furthermore, sero-positivity is investigated for each serological marker according to the different districts (Figure 35). All districts show high sero-positivity for Ags LSA3-RE, Pf MSP1-19, CSP, GLURP, Pf GLURP R2 and SALSA2. For those Ags the highest sero-positivity levels are seen in districts Oyadao, Lum Phat and Voen Sai.

For some Ags (CSP, SALSA2, SALIV2, PmCSP, Pv like CSP and PvVK210 CSP) there is no significant difference between the sero-positivity for those Ags (Kruskal-Wallis test, p>0.05). For other Ags there is a significant difference between the seven districts in sero-prevalence (Kruskal-Wallis test, p<0.05).



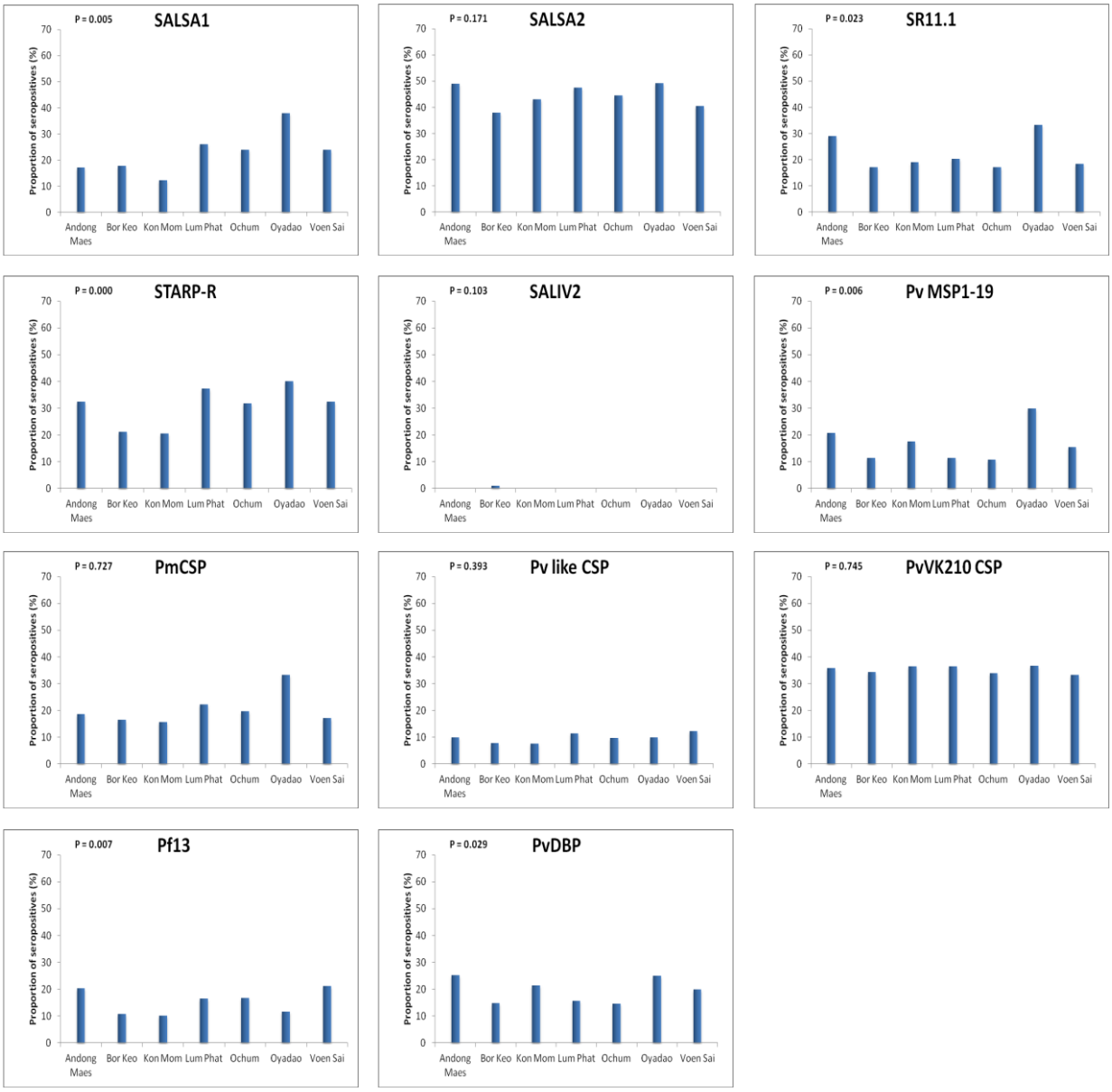


Figure 35: ΔMFI (mean) and sero-prevalence calculated per district. The bars stand for the sero-positivity (%). The p-value is based on the Kruskal-Wallis test.

5 Discussion

5.1 Advantages of the multiplex assay

In areas with a low prevalence and incidence of malaria transmission, such as Cambodia, detection of parasitological indicators of the transmission intensity can be difficult. Serology allows for a more sensitive detection of malaria exposure and can be used as a proxy of malaria transmission [38]. Therefore, recently a multiplex assay has been developed for the simultaneous detection of multiple Abs based on the Luminex-technology. The general objective of this thesis was therefore to implement an existing assay based on the Luminex-technology, for the detection of Abs against malaria parasites in blood samples within the MalaResT project. This will makes it possible to detect twenty malaria specific Abs simultaneously instead of each Ab separately as in the ELISA.

For this assay fourteen malaria specific peptides, one *An. gambiae* salivary Ag and five malaria specific recombinant proteins were chosen. Previous studies on malaria done by Ambrosino et al [27], Sarr et al [59], and Khaireh et al [58] have already proven that the multiplex immunoassay is useful in serology, but were limited to a maximum of respectively 15, 13 and 17 Ags.

The multiplex immunoassay used to study other diseases or infections also proved that this kind of assay is useful for serology [67–69]. Advantages have been seen in the capacity to test more than one serological marker, as well as in the rapid analysis and higher throughput [67–69]. The multiplex immunoassay performed on malaria in this study is the first performed in Cambodia and the first study that includes twenty different peptides simultaneously. Several studies have developed the multiplex assay for serology and have proven that the results obtained with the multiplex are similar and just as sensitive in comparison with those obtained with ELISA [21, 27, 70]. The advantages of the multiplex assay when compared to the ELISA are that the multiplex assay decreases time and costs. A major reason for testing more than one Ag instead of just one Ag is that not every individual responds to the same Ags in the same way and that the chances of measuring Ab responses are higher with more Ags. According to Sarr et al [59] assessment of the force of infection is more precise when using more than one Ag, sero-positivity to several Ags reflects cumulative exposure to malaria infection. Even though the Luminex materials and machines have a high price compared to the ELISA, costs are reduced because research of more Abs can be simultaneously performed, instead of each Ab separately. The difference is that the lower costs of the ELISA machines make them more widespread than the Luminex machines [27]. Another advantage of the Luminex is that only a small volume of serum is required for the screening of different Ags in large populations [27].

5.2 Positive sera and Ag selection in ELISA

For positive sera selection, sensitivity and specificity of the ELISA depend on the quality of used sera, which should contain Abs that respond specifically to the coated Ag. Therefore, seventeen malaria positive sera from Cambodia were used. Preferably, a high concentration of Abs should be present in the positive control to ensure a reaction only occurs with the coated Ags [71]. To control for unspecific reactions with reagents in the ELISA, the ELISA plates were divided into two different parts (Ag-coated-site and a non-coated site) (see Figure 1 ANNEX 1) and to control for unspecific reactions in the multiplex assay we added an extra beads-set coupled with BSA. To select the positive sera for this study, REF_P_sera (pooled sera from five *P. falciparum* and *P. vivax* infected patients) and as a negative control pooled sera from five non-infected control group both diluted at 1:400 were used [38].

The selection of positive controls used in ELISAs or multiplex assays is not described in many papers. Therefore, it is difficult to compare the current selection with other studies, as the selection of the positive controls is a crucial step within studies based on Ab detection.

In this research six positive sera were selected (dilution 1:400) to use for the pool of sera (dilution 1:100). A selection of the twenty four Ags needed to be made as only twenty different beads were available for malarial Abs. For the Ag selection the Ags, Saliv1, TRAP1, TRAP2 and PvVK247-CSP gave too low results in the ELISA to select a good positive control serum (see Figure 15 paragraph 4.1). The

low results can be explained, since the used Ags were chosen according to the paper described by Ambrosino et al [27] and these Ags were tested in Africa. Based on the results in the paper of Ambrosino et al [27] a strong signal was observed with SALIV1 when tested on serum from African people but a low signal was observed by using serum from travellers, and the remaining three Ags gave low results on both African people and travelers [27]. As SALIV1 and SALIV2 are developed from the genome of an African vector *An. gambiae* [72] it is not surprising that the response to these Ags is very low in Cambodian malaria patients.
In our reasearch finding a good positive control on the PvVK247-CSP was however expected. The ELISA gave very low results, which does not imply that there are no proper positive controls to be found for those Ags. While seventeen positive sera were used to select the right sera for each Ags, it is possible that a proper positive control will be found after testing more *Plasmodium* positive sera. Unfortunately, this is time-consuming. In areas with a low intensity of malaria transmission, such as Cambodia, there is only a certain amount of samples available as in routine diagnosis suspected malaria cases do not need to provide serum samples. Moreover, it takes a lot of serum to screen them for all Ags. It is also possible that there is another CSP variant in Cambodia, which makes it almost impossible to find a good positive control for PvVK247-CSP. Some of the other Ags (STARP-R, SALIV2, Pv like CSP) were included in the assay even though the ELISA gave very low results, and they did not have a good positive control. For STARP-R a strong respons in the screened samples was found, therefore it might have been better to include PvVK247-CSP as well. This can be explored in the next phase of this study.
Looking at Figure 15 paragraph 4.1, TRAP1 gave the same result as STARP-R, while TRAP2 gave lower results on the ELISA. The reason that TRAP1 and TRAP2 were removed from the research is that those also gave low or no results in the research done by Ambrosino et al [27] and STARP-R had some strong results in this research. Furthermore, a study performed in Thailand [73] gives information about the occurrence of STARP-R in low endemic areas. They confirmed that STARP-R is a potential vaccine candidate, which indicates that in this region, Abs are being developed against STARP-R. Therefore, STARP-R was interesting to add in this research.

5.3 Testing of the beads-coupling

Obtained results showed that all Ags were effectively coupled to the beads and gave good results, but some of them gave unexpectedly low results based on the ELISA. Low signals were observed with Pf13 which can be explained, since there was no strong positive serum for this protein found with the ELISA (ANNEX III). For Pv MSP1-19, the difference between ELISA and Luminex may be caused, because the epitopes recognized by ELISA are not the same as the ones presented after coupling of the beads or that a lower amount of proteins binds to the beads. The difference observed between the coupling to the wells (ELISA) and the coupling to the beads, due to alternations and conformations in presenting epitopes during the ELISA and the multiplex assay, is a limitation of the multiplex technique [27, 59].
In the paper of Ondigo et al [74], the use of proper amounts of Ags is essential in controlling the surface density of the malaria Ags on the bead surface. They indicated that sub-optimal coating and low activity will appear when using too small amounts of Ags. But that too much Ags will lead to precipitation and aggregation of the beads which can affect the Ab binding on the surface of the beads. To study if this is what happened to the beads-coupling in this thesis it would be interesting to do an experiment with different amounts of Ag for the binding to the beads. In this thesis similar amounts of Ags as in the studies of Ambrosino et al [27] and Sarr et al [59] are used for coupling of the beads.

5.4 Comparison between the monoplex vs multiplex and 1000 vs 2000 beads

According to the obtained results, there is a good correlation between the monoplex and the multiplex assay. However, the Ags GLURP, Pf GLURP R2, SALIV2 and Pv DBP gave higher results in the multiplex than in the monoplex assay. In a paper written by Fouda et al [21] there is a decrease in the

multiplex compared to the monoplex assay at high plasma dilutions, which is in contrast with our results. The reason for an increase or decrease in MFI in a multiplex or monoplex assay is not clear.
For the Ags LSA1-41, LSA1-J and Pf MSP1-19, lower results are found in the 1:100 and 1:200 dilution compared to the 1:400 dilution. This gives an abnormal curve compared to the remaining Ags. A possible explanation is that the efficiency of the assay drops when Ab levels are too high.
In this thesis we have established that the use of a lower number of beads (1000 beads/Ag/well) is just as effective as the use of 2000 beads/Ag/well. Looking at the results (see Figure 20 and Table 10 in paragraph 4.4) a minimal variation between the use of 1000 and 2000 beads/Ag/well is shown. If the costs of the use of 1000 and 2000 beads/Ag/well are roughly calculated, it is clear that 1/3th of the costs can be saved by using 1000 beads/Ag/well. Where the assay will cost approximately €6.4 per sample in duplicate when using 1000 beads/Ag/well, for 2000 beads/Ag/well these costs will be approximately €9.6 per sample in duplicate.
Standard testing protocols for the multiplex bead-based assay recommend 5000 beads/Ag/well. Ondigo et al [74] also established that with 1000 beads/Ag/well similar results could be obtained as compared to 5000 beads/Ag/well. This is in concordance with our study.

5.5 Comparison between multiplex assay and ELISA

In this thesis, the multiplex assay and the ELISA were compared to each other at a dilution of 1:400. Some of the Ags (SALSA1, SR11.1 and Pv MSP1-19) gave low results in percent positivity with the multiplex assay and high results in the ELISA. A reason for the differences in percent positivity can be that the way how the epitopes are presented in the ELISA is not the same as the way they are presented after coupling of the beads. In Ambrosino et al [27] the Ags that responded low in the multiplex assay, such as LSA3-RE and SALIV1, were also compared to the results previously obtained with the ELISA. Similar to what was observed for LSA3-RE and SALIV1 in Ambrosino et al we also see a discrepancy between the percent positivity obtained in the multiplex assay and the ELISA for several Ags (SALSA1, SR11.1 and PvMSP1-19).
The other Ags (STARP-R, SALIV2, Pv like CSP, PvVK210-CSP and Pf13) that gave low results in the multiplex assay during this thesis can be explained, because they also gave low results in the ELISA, and therefore no good positive control was selected. Furthermore, the remaining Ags gave similar results in the multiplex and ELISA assay.

5.6 Interassay reproducibility

In this study a small experiment was performed to test whether the results are accurate and reproducible from one assay to another. The results show that there is not a big difference seen between the two different plates and according to the R^2 and p-values a good correlation between the assays on the two different plates is seen. The reproducibility of multiplex bead arrays has also been tested in other studies [21, 27, 70, 74, 75] which also showed that the results are accurate and reproducible between different assays. As mentioned in paragraph 3.10, a quality control of the high positive controls and a validation of the samples is done by comparing each sample with its duplicate. After the quality control and the sample validation from a total of 72 plates only two whole plates (2a and 2b) are removed, indicating that the assay has a good reproducibility.

5.7 Determining the percent positivity for each serological marker and between different groups in the baseline survey

Looking at the number of Ags recognized by PCR positive and negative individuals, most of the Pf positive people respond to LSA3-RE, CSP, Pf MSP1-19, GLURP, SALSA2 and Pf GLURP R2 and less people respond to the remaining Ags. For Pf MSP1-19 this is probably due to the long half life (6.7 years) [76]. Pv MSP1-19 has a long half life as well (≈ 50 years) [77], but according to the results obtained in this research most of the people responded to PvVK210 CSP and Pv DBP instead of Pv MSP1-19. In this case the long half life is not an explanation for all Ags. It might be possible that PvMSP1-19 has a lower antigenicity than PvVK210 CSP and Pv DBP. In the case of *P. falciparum* a lot

of people responded to GLURP, which is known to have a short half live [78]. An explanation for the high response to GLURP can be that the antigenicity of this Ag is very high, meaning that each time a person comes in contact with this Ag, high levels of Abs are produced.
Figure 31 in paragraph 4.11 shows the PCR-positive and -negative status in relation to the number of Ags of Pf and Pv. Most of the Pf positive people responded to nine Ags and most of the Pf negative people to four Ags. Looking at Pv most of the Pv positive people responded to one Ag and most of the Pv negative people responded to zero Ags (some responders are seen for two, three and four Ags). This makes clear that a response to a higher number of Ags was observed in PCR positive samples when compared to PCR negative samples.
Some of the individuals tested negative in PCR but still responded to several Ags. This is due to the fact that PCR prevalence data provides a snapshot of the endemicity at a certain moment, while the multiplex assay provides a picture of the endemicity over a larger period. This means it is possible that people who are tested negative in PCR still have Abs present from an earlier malaria infection depending on the Ab half-life. When comparing the serology result of PCR positive and negative *P. malariae* individuals, a significant difference is seen for PmCSP, Pf GLURP R2 and PfCSP. At first glance this seems strange, as you would only expect a difference for PmCSP. There is however a logical explanation, since there are only four Pm PCR positive individuals, of which only one was PCR-positive for Pm, whereas the other three were mixed infections (PvPm, PfPm and PfPvPm). A similar event is seen for *P. vivax* individuals were a significant difference is seen for Pv MSP1-19, Pv DBP, Pf MSP1-19, LSA3-RE, Pf GLURP R2, GLURP and LSA1-41, which also seems strange, since you would expect the significant differences on all Pv samples and not on the Pf samples. As seen in the *P. malaria* individuals there are thirty-two Pv PCR positive individuals, of which twenty-two are positive for Pv only, whereas the other ten were mixed infections (eight positive for PfPv, one for PvPm and one for PfPvPm). This similar event is not seen on *P. falciparum* individual despite the fact that from the twenty-five Pf PCR positive individuals ten were mixed infections (PfPV, PfPm and PfPvPm).

According to the sero-prevalence per Ag the results suggest that sero-positivity to Ags increases with age, which makes clear that sero-positivity to different Ags reflects frequent exposure to malaria infection. Looking at gender, for both Pf and Pv no significant difference is observed between men and women. Given that in Cambodia mostly men are considered a risk group, it is strange that men and women have a similar sero-positivity. However, in a study performed in Cambodia by Incardona et al [79] for example, based on microscopy, there is a difference in malaria prevalence, between men and women. Due to the work activities of men (farming, forested activities including woodcutting, hunting, gemstone mining), and because they work with the upper body uncovered, men are expected to be more exposed to malaria mosquitoes than women.
High sero-positivity is seen in three (Oyadao, Lum Path and Voen Sai) of the seven different districts, especially for the Ags LSA3-RE, Pf MSP1-19, CSP, GLURP, Pf GLURP R2 and PvVK210 CSP. At geographical level Cook et al [38] have shown differences in the force of infection, which makes it logical that differences between different districts are seen in our study. It would be interesting to explore the reason for these differences in future studies.

5.8 Comments on the use of the multiplex assay in the future

Next year this assay will be performed on the remaining three surveys of the large scale repellent project. While Ags SALIV2, PV like CSP, SALSA1, PvVK210-CSP, PmCSP and SR11.1 gave very low results during the testing phases, only SALIV2 and Pv Like CSP gave low or even no responses in the screened blood samples. On the other hand PvVK210-CSP, SALSA1, SR11.1 and Pm CSP gave surprising results in sero-prevalence even though there was not a very strong control serum found for those Ags. Therefore, a suggestion for the use of this assay next year will be to remove the Ags SALIV2 and pv like CSP, because the responses of those Ags were too low. The Ag PvVK210-CSP resulted in a surprisingly high sero-prevalence as compared to the results obtained in the first screening of the control sera. PvVK210 and PvVK247 are sometimes found in mosquitoes in and around Southeast Asia in Indonesia, Papua New Guinea, Bangladesh, Vietnam and Cambodia [80]. A

paper that has found information about the occurrence of PvVK210-CSP and PvVK247-CSP is written by van Bortel et al [81]. In this paper they found small numbers of PvVK210-CSP and PvVK247-CSP in mosquitoes collected in the forested areas in central Vietnam. Therefore it would be interesting to add PvVK247-CSP to this assay as well next year.
There was a high sero-prevalence for PmCSP seen in Cambodia. This is an Ag that definitely should be retained in the assay. Unfortunately, only a limited amount of the serum 3215/0013/04 that gave strong results in the ELISA, was available and finished after the testing phases. Therefore, it would be interesting to see if another strong positive control serum could be found for this Ag.
SALIV1 and SALIV2 Ag are derived from *An. gambiae* saliva glands. As those are the only salivary gland Abs available it was interesting to evaluate the immune responses against mosquito Ags. Only SALIV2 was added to this study, since this Ag gave one high response on the first screening of the control sera in the ELISA and SALIV1 gave no response at all. If this approach would have resulted in a high sero-positivity, this could have given an indication of the exposure to vector bites [27]. The fact that *An. gambiae* (an African malaria vector) does not occur in Southeast Asia can explain why the percent positivity and the sero-prevalence for SALIV2 were extremely low. Therefore it will be better to remove this Ag from the assay. Another Ag should be developed, derived from the main malaria vectors in Cambodia, namely *An. dirus* and *An. minimus* [12, 81].
Due to time constraints and as the most important aim of this thesis was to implement and optimize the assay, an in-depth data analysis on the results of the blood sample screening has not yet been performed. These data should however be further explored before continuing the sample analysis of the other surveys. When looking at the number of people that are sero-positive per Ag it would be interesting to analyze which Ags mostly react together to know which Ags suit as the best indicator for sero-positivity. For example, if one Ag is always embedded in another one, then it would not be necessary to test the first one as the second one will be enough to determine the sero-positivity status of the individual.

In the future this assay can be useful in other studies or fields. As we are performing this research in a country where the prevalence is decreasing and is moving towards malaria elimination, it is more difficult to collect PCR positive samples, because there will be less and less parasites. This assay can be useful in this respect to measure the efficiency of the malaria control program. Therefore, this assay could be useful in future studies to measure malaria control efficiency and continuing presence of malaria.

6 Conclusion

The multiplex bead-based immunoassay was successfully implemented in the molecular malaria unit of the IPC. This multiplex assay is a reliable fast method and useful tool for simultaneous detection of Ab against *Plasmodium* Ags used as potential markers of malaria transmission in blood samples. In areas with a low transmission, such as Cambodia, detection of parasitological indicators of the transmission can be labor-intensive and time-consuming. Therefore, serological measurements to different malaria Ags are needed for measuring short term and small scale variations in the malaria transmission intensity. The assay was used to screen 1440 samples from the baseline survey of the large scale repellent project, which took about three weeks. The results in this thesis show that this assay is very time-efficient and very informative.

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8 Annexes

8.1 ANNEX 1

Institut Pasteur du Cambodge	ELISA for plasmodium antibodies detection Selection of controls serums for luminex assays		SOP AP_SER001 V. 1.0
	Malaria Molecular Epidemiology Unit	Page 1/7	07/05/2013

Written by	Verified by	Validated by
• Karen Kerkhof	• Lydie Canier	• Didier Ménard

SOP versioning:

Version #	Date	Comments
V 1.0	07/05/2013	

Table of Content

1. Aim and application

The ELISA Is used to screen serum samples from malaria positive patients for the presence of antibodies to the different antigens in order to identify serum samples that can be used as positive controls in the Luminex assay. As ITM has extensive experience with PfGLURP-R2 recombinant protein in the ELISA and possesses a control serum for this assay, this combination of antigen and serum will be used as control for all plates in this ELISA protocol.

2. Reference material

Cook, J., Speybroeck, N., Sochanta, T., Somony, H., Sokny, M., Claes, F., Lemmens, K., Theisen, M., Soares, I. S., D'Alessandro, U., et al. (2012). Sero-epidemiological evaluation of changes in Plasmodium falciparum and Plasmodium vivax transmission patterns over the rainy season in Cambodia. Malaria journal *11*, 86–97.

Drakeley, C. J., Corran, P. H., Coleman, P. G., Tongren, J. E., McDonald, S. L. R., Carneiro, I., Malima, R., Lusingu, J., Manjurano, A., Nkya, W. M. M., et al. (2005). Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. Proceedings of the National Academy of Sciences of the United States of America *102*, 5108–5113.

3. Hygiene and security

Laboratory personnel should always wear a laboratory coat and latex of nitrile gloves when working on the ELISA. After use 70% Ethanol must be used to decontaminate the work surface.

4. Reagents and consumables

4.1. Equipment, Materials & Reagents

- Antigens:

Recombinant proteins (1mL	sequences	Company Ags
Glurp	EDKNEKGQHEIVEVEEILC	GeneCust
BSA (background)		Sigma-Aldrich
CSP	NANPNANPNANPNANPNVDPNVDPC	GeneCust
Glurp P3 (2mL)	EPLEPFPTQIHKDYKC	GeneCust
Lsa1-41	LAKEKLQEQQSDLEQERLAKEKLQEQQSDLEQERLAKEKEKLQ	GeneCust
Lsa1-J	ERRAKEKLQEQQSDLEQRKADTKKC	GeneCust
Lsa3-NR2	VLEESQVNDDIFNSLVKSVQQEQQHNVC	GeneCust
Lsa3-RE (2mL)	VESVAPSVEESVAPSVEESVAENVEESVC	GeneCust
Salsa 1	SAEKKDEKEASEQGEESHKKENSQESAC	GeneCust
Salsa 2	NGKDDVKEEKKTNEKKDDGKTDKVQEKVLEKSPKC	GeneCust
SR11.1	EEVVEELIEEVIPEELVLC	GeneCust
Starp R	STDNNNTKTISTDNNNTKTIC	GeneCust
Saliv 2	ATFKGERFCTLCDTRHFCECKETREPLC	GeneCust
Pm CSP	GNAAGNAAGNDAGNAAGNAAGNAAGNAAGNAAC	GeneCust
Pv-like-CSP	APGANQEGGAAAPGANQEGGAAAPGANQEGGAAC	GeneCust
PvVK210 CSP	DGQPAGDRAAGQPAGDRADGQPAGDRADGQPAGC	GeneCust

Table 1: Beads numbers belonging to the peptides; amount of peptides needed for bead coupling [56].

Recombinant proteins (1mL	Company Ags
Pf GLURP R2	ITM-Antwerp
Pf MSP1-19	IP-Paris
Pv MSP1-19	IP-Paris
Pf13	IP-Paris
Pv DBP	IP-Paris

Table 2: Beads numbers belonging to the recombinant proteins; amount of proteins needed for bead coupling [56].

- Reagents:

Reagents	Reference number	Company
ABTS-powder	11 112 597 001	ROCHE
ABTS-tablets	11 112 422 001	ROCHE
NaCL	27 810.295	PROLABO
$NaH_2PO_4.H_2O$	28 013.264	PROLABO
$Na_2HPO_4.2H_2O$	S0876	Sigma-Aldrich
Skimmed Milkpowder (1% fat)	-	-
Goat anti-human IgG conjugate	109-035-003	Jackson ImmunoResearch
dPBS	14190-094	GIBCO
Tween 20, $C_{58}H_{114}O_{26}$	P7949	Sigma-Aldrich

Table 3: Overview of the materials and the company.

- Test sera: Sera that are suspected to react positive to the antigens:
  - Test Pf: sera that are suspected to react positive to the Pf related antigens
  - Test Pv: sera that are suspected to react positive to the Pv related antigens
  - Test Pm: sera that are suspected to react positive to the Pm related antigens

- Materials:

Materials	Company
Micro-pipettes (10; 200; 1000 µl)	Eppendorf
Multichannel pipettes (300 µl)	Eppendorf
Pipette filter tips (10; 200;1000 µl)	Neptune
Vortex	-
ELISA-reader (with filter of 405-415nm)	-
ELISA-washer	-
High binding, flat bottom plates	COSTAR

Table 4: Overview of the materials and the company.

- Extra materials: Plate sealers, Duran bottle (1L), Eppendorf tubes (1.5mL), Tinfoil, Falcon tubes (50mL), tubes (15mL), Absorbent pads or paper towels, Waste receptacle, Reagent reservoirs, Refrigerator (2-8°C), freezer (-80°C ± 5°C & -20°C ± 5°C), Disposable gloves, Lab coat & sleeve protectors, Racks for cryo- & eppendorf tubes.

4.2. Reagents and Buffers preparation

Reagents	Conc.	PH	Store	Preparation
PBS-tween	0.01M	7.4	4°C	1L PBS 0.01M + 500µL Tween 20 (0.05%)
PBS-Blotto	0.01M	7.4	4°C	Fill a beaker with 0.7L dH_2O NaCl 11.7gr + $NaH_2PO_4.H_2O$ 0.2gr + $Na_2HPO_4.2H_2O$ 1.44 gr + skimmed milkpowder (1%) 10gr → add the products in the dH_2O → stir solution 30 min. Dilute until exactly 1L with dH_2O. Let sediment during one night at 4°C or centrifuge the solution 20 minutes on 3000 RPM (1855 g), no brake. Measure pH! (pH 7.4 +/- 2)
PBS-Blotto-Tween	0.01M	7.4	4°C	250 mL PBS-Blotto + 125µL Tween 20 (0.05%)
Buffer ABTS			4°C	16.7 g powder in 1L pure water. (storage 3 months)
Substrate solution			4°C	Dissolve 1 tablet of ABTS in 100mL ABTS buffer (storage 1 week).

Table 5: Preparation of the Reagents and Buffers.

5. Procedure

5.1. Coating of the antigens

1. Starting from a 1mg/ml solution
   a. For the peptides, e.g. if starting from 1 mg antigen, than add 1 ml H_20 (Milli-Q water) If not all the peptides dissolve, add TFA up to a maximum concentration of 0.5% (trifluoroacetic) (or 5 µl in the 1000 µl H_2O), or add another substance to facilitate the dissolving of the peptides.or 1% NH4OH
2. Dilute the antigen in PBS-buffer to a final concentration of 2µg/ml (dilution 1/500 if starting from a 1mg/ml solution).
   a. Prepare 3 ml PfGLURP-R2 (6 µl of undiluted antigen + 3 ml PBS-buffer)
   b. Prepare 8 ml of test antigen (4 µl of undilated antigen + 8 ml PBS-buffer)
3. Fill column 1 and Row A & B of column 2 with 200µl/well of PfGLURP-R2. Fill the rest of the wells of the left half of the plate with 200 µl/well of the test antigen dilution ('coated' side), leave the right half of the plate empty (antigen negative control, 'uncoated' side) (Figure 1).
4. Incubate overnight at 4°C.

5.2. Saturation

1. Remove the antigen solution
2. Add 300 µl PBS-Blotto per well, incubate for 1 hour at ambient temperature.
3. During incubation → prepare the controls (strong positive (+++), weak positive (+) and Negative (-)) (dilution 1/400)
   - E.g. positive control (+++) or negative control (-) for 1 plate: 8 controls of each per plate: 8*200µl/well= 1.6 ml (Prepare 2 ml in a 3.5 ml tube)
     - Fill each-tube with 2 mL PBS-Blotto
     - Vortex each control
     - Centrifugation for 5 min/ max rpm
     - Add 5 µl of the control in the Falcon tubes (dilution 1/400)
   - E.g. test serum or weak positive control (+) for 1 plate: 4 serum samples or controls of each per plate: 4*200µl/well = 0.8 ml (Prepare 1 ml in a 3.5 ml tube)
     - Fill each-tube with 1 mL PBS-Blotto
     - Vortex each control
     - Centrifugation for 5 min/ max rpm
     - Add 2.5 µl of the control in the Falcon tubes (dilution 1/400)

5.3. Execution of the ELISA test

1. Remove the PBS-Blotto

2. Wash: Wash the plate 3 times (manually 1 minute, automatically 1 second, choose program malarest3) with 300 µl of PBS-Tween/well. (If you want to stop at this step, you can cover the plate with sealing tape and store frozen at -70°C. Before use then thaw the plate and allow it to come at ambient temperature. Then wash it again before using it.)
3. Vortex the tubes of the diluted controls and serum samples:
   - Add 200 µl of serum sample in every well according to the plate configuration in figure 2 (ANNEX III).
   - Use 200 µl PBS-Botto/well as conjugate control (blanco)
   - Incubate for 1 hour at ambient temperature
4. Prepare the conjugate: Dilute the conjugate at 1/50000 in PBS-Tween
   - E.g. for 1 plate: PBS-Tween: 200 µl/well * 100(96 well plate) = 20 mL needed
   - Add 10 µl (1:2 conjugate) in 10 mL PBS-Tween = 1/2000
   - Take 1 mL of the 1/2000 dilution and add in 25 mL PBS-Tween = 1:50000
5. Wash: Wash the plate 3 times (manually 1 minute, automatically 1 second, choose program malarest3) with 300 µl of PBS-Tween/well.
6. Add conjugate 200 µl/well to every well and incubate for 1hour at ambient temperature
7. Prepare the substrate (200µl/wel)
   - E.g. for 1 plate: 200 µl/well * 100 = 20 mL (substrate needed)
   - To make the ABTS-buffer: Add 16,7 g ABTS powderin 1 liter Milli-ρ H_2O . Store at 4°C for maximum 3 months
   - Substrate solution: Dissolve 1 tablet in 100 mL ABTS-Buffer (This solution can be used for one week only).
   - Seal with tinfoil (incubate at ambient temperature until diluted)
8. Wash: Wash the plate 5 times (manually 1 minute, automatically 1 second, choose program malarest5) with 300 µl of PBS-Tween/well.
9. Add 200 µl/well of ABTS-substrate to every well and incubate for 1hour at ambient temperature.
10. Reading: Read optical density (O.D.) at 405 nm (or 415 nm if 405nm is not available) after shaking the plate in the ELISA reader for 10 seconds at medium speed.
    - Start computer
    - Start reader → password → press enter
    - Open program microplate manager 5.2.1
      - File
      - New endpoint protocol
      - Measurement filter 415nm
      - Place the ELISA plate inside the reader
      - Run
    - Copy paste the results into excell

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5.4. Data analysis

1. For each test sample, subtract the O.D. obtained in the antigen free well from the O.D. obtained in the antigen containing well.
2. Calculate the COD (corrected OD-values (the coated wells – non coated wells and then take the average).
3. Calculate the percent positivity.
4. For all these calculations, use the EXCEL format "EMPTY_TEMPLATE_ELISA". This template will do all the calculations.

5.5. Plate setup

Figure 1: ELISA plate coating

	Ag						non coated (negative control)					
	1	2	3	4	5	6	7	8	9	10	11	12
A	Pf GLURP R2	Pf GLURP R2	Test AG	Test AG	Test AG	Test AG						
B	Pf GLURP R2	Pf GLURP R2	Test AG	Test AG	Test AG	Test AG						
C	Pf GLURP R2	Test-AG	Test AG	Test AG	Test AG	Test AG						
D	Pf GLURP R2	Test-AG	Test AG	Test AG	Test AG	Test AG						
E	Pf GLURP R2	Test AG	Test AG	Test AG	Test AG	Test AG						
F	Pf GLURP R2	Test AG	Test AG	Test AG	Test AG	Test AG						
G	Pf GLURP R2	Test AG	Test AG	Test AG	Test AG	Test AG						
H	Pf GLURP R2	Test AG	Test AG	Test AG	Test AG	Test AG						

Figure 2: ELISA plate configuration

	Ag						non coated (negative control)					
	1	2	3	4	5	6	7	8	9	10	11	12
A	+++	BL	2	6	10	14	+++	BL	2	6	10	14
B	+++	BL	2	6	10	14	+++	BL	2	6	10	14
C	+++	-	3	7	11	15	+++	-	3	7	11	15
D	+++	-	3	7	11	15	+++	-	3	7	11	15
E	+	BL	4	8	12	16	+	BL	4	8	12	16
F	+	BL	4	8	12	16	+	BL	4	8	12	16
G	-	1	5	9	13	17	-	1	5	9	13	17
H	-	1	5	9	13	17	-	1	5	9	13	17

+++: ITM strong positive serum
+: ITM weak positive serum
-: ITM negative serum
BL: PBS-BLOTTO
Numbers 1-17: IPC test serum

8.2 ANNEX 2 (MAGPIX SOP)

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Written by	Verified by	Validated by
• Karen Kerkhof	• Lydie Canier	• Didier Ménard

SOP versioning:

Version #	Date	Comments
V 1.0	07/05/2013	

Table of Content

1. Object

The test is used to simultaneously detect multiple Ab against different *Plasmodium* Ags in serological samples.

2. Definitions

The MAGPIX is designed for the use of MagPlex polystyrene paramagnetic microspheres (beads) as a substrate. The principle of this technique is based on coating different magnetic beads with a specific antigen so when multiplexed in one assay. The different beads can capture and quantify various antibodies in one blood sample. The magnetic beads will be kept in the wells by using a magnetic separator during the protocol.

3. Reference

Ambrosino, E., Dumoulin, C., Orlandi-Pradines, E., Remoue, F., Toure-Baldé, A., Tall, A., Sarr, J. B., Poinsignon, A., Sokhna, C., Puget, K., et al. (2010). A multiplex assay for the simultaneous detection of antibodies against 15 Plasmodium falciparum and Anopheles gambiae saliva antigens. Malaria journal *9*, 317–328.

Sarr, J. B., Orlandi-Pradines, E., Fortin, S., Sow, C., Cornelie, S., Rogerie, F., Guindo, S., Konate, L., Fusaï, T., Riveau, G., et al. (2011). Assessment of exposure to Plasmodium falciparum transmission in a low endemicity area by using multiplex fluorescent microsphere-based serological assays. Parasites & vectors *4*, 212–219.

Khaireh, B.A., Briolant, S., Pascual, A., Mokrane, M., Machault, V., Travaille, C., Khaireh, M.A., Farah, I.H., Ali, H.M., Abdi, H.M.A., et al. (2012). Plasmodium vivax and Plasmodium falciparum infections in the Republic of Djibouti: evaluation of their prevalence and potential determinants. Doi:10.1186/1475-2875-11-395.

4. Hygiene and security

Sodium Azide is a toxic reagent used as a preservative of the buffers used in this protocol. It may cause liver and kidney damage or can be fatal if inhaled, absorbed through skin or ingested.

Laboratory personnel should always wear a laboratory coat, eye protection and latex of nitrile gloves when using Sodium Azide. After use 70% Ethanol must be used to decontaminate the work surface.

5. Reagents and consumables

5.1. Equipment, Materials & Reagents

- Antigens:

Peptides

Bio-Plex COOH Bead numbers	Recombinant proteins (1mL dilution)	sequences	Ci (µg/mL)	Vi (µg/mL)	Company Ags	Company Beads
012	Glurp	EDKNEKGQHEIVEVEEILC	4000	5,0	GeneCust	Luminex Corporation
013	BSA (background)		4000	5,0	Sigma-Aldrich	Luminex Corporation
014	CSP	NANPNANPNANPNANPNVDPNVDPC	4000	5,0	GeneCust	Luminex Corporation
015	Glurp P3 (2mL)	EPLEPFPTQIHKDYKC	2000	10,0	GeneCust	Luminex Corporation
018	Lsa1-41	LAKEKLQEQQSDLEQERLAKEKLQEQQSDLEQERLAKEKEKLQC	4000	5,0	GeneCust	Luminex Corporation
019	Lsa1-J	ERRAKEKLQEQQSDLEQRKADTKKC	4000	5,0	GeneCust	Luminex Corporation
020	Lsa3-NR2	VLEESQVNDDIFNSLVKSVQQEQQHNVC	4000	5,0	GeneCust	Luminex Corporation
021	Lsa3-RE (2mL)	VESVAPSVEESVAPSVEESVAENVEESVC	2000	10,0	GeneCust	Luminex Corporation
026	Salsa 1	SAEKKDEKEASEQGEESHKKENSQESAC	4000	5,0	GeneCust	Luminex Corporation
027	Salsa 2	NGKDDVKEEKKTNEKKDDGKTDKVQEKVLEKSPKC	4000	5,0	GeneCust	Luminex Corporation
028	SR11.1	EEVVEELIEEVIPEELVLC	4000	5,0	GeneCust	Luminex Corporation
029	Starp R	STDNNNTKTISTDNNNTKTIC	4000	5,0	GeneCust	Luminex Corporation
030	Salv 2	ATFKGERFCTLCDTRHFCEQKETREPLC	4000	5,0	GeneCust	Luminex Corporation
034	Pm CSP	GNAAGNAAGNDAGNAAGNAAGNAAGNAAGNAAC	4000	5,0	GeneCust	Luminex Corporation
035	Pv-like-CSP	APGANQEGGAAAPGANQEGGAAAPGANQEGGAAC	4000	5,0	GeneCust	Luminex Corporation
036	PvVK210 CSP	DGQPAGDRAAGQPAGDRADGQPAGDRADGQPAGC	4000	5,0	GeneCust	Luminex Corporation

Table 1: Beads numbers belonging to the peptides; amount of peptides needed for bead coupling [56].

Recombinant proteins

Bio-Plex COOH Bead numbers	Recombinant proteins (1mL	Ci (µg/mL)	Vi (µg/mL)	Company Ags	Company Beads
022	Pf GLURP R2	1000	20,0	ITM-Antwerp	Luminex Corporation
025	Pf MSP1-19	3000	7,5	IP-Paris	Luminex Corporation
033	Pv MSP1-19	2000	10,0	IP-Paris	Luminex Corporation
037	Pf13	2500	8,0	IP-Paris	Luminex Corporation
038	Pv DBP	150	133,33	IP-Paris	Luminex Corporation

Table 2: Beads numbers belonging to the recombinant proteins; amount of proteins needed for bead coupling [56].

- Reagents:

Reagents	Reference number	Company
NaH_2PO_4	28 013.264	PROLABO
MES	M2933	Sigma-Aldrich
dPBS	14190-094	GIBCO
BSA	A4503	Sigma-Aldrich
CR	RG-MDL-1	Charles River Laboratories
EDC	03449 (LOT# BCBF5908V)	Perbio Science/ Thermofisher
Sulfo-NHS	56485 (LOT# BCBJ4456V)	Perbio Science/ Thermofisher
Tween (20)	P7949	Sigma-Aldrich
Sodium azide	S2002	Sigma-Aldrich
Calibration kit for MAGPIX	MPX-Cal-K25 (LOT# B24327)	Luminex Corporation
Validation kit for MAGPIX	MPX-PVER-K25 (LOT# B24054)	Luminex Corporation
R-Phycoerythrin+-conjugated AffiniPure F(ab')2 (Goat anti-human IgG)	109-116-170	Jackson Immuno Research Laboratories

Table 3: Overview of the materials and the company.

- Materials:

Materials	Company
Micro-pipettes (10; 200; 1000 µl)	Eppendorf
Multichannel pipettes (300 µl)	Eppendorf
Pipette tips (10; 200;1000 µl)	Neptune
Vortex	-
Plate shaker	-
Sonicator	Cole-Parmer
Magnetic separator	Luminex Corporation
BioRad Bio-Plex MAGPIX	Luminex Corporation
96-well plates (Nunc roundbottom plates)	Sigma-Aldrich

Table 4: Overview of the materials and the company.

- Extra materials: Plate sealers, Duran bottle (1L), Eppendorf tubes (1.5mL), Tinfoil, Falcon tubes (50mL), tubes (15mL), Absorbent pads or paper towels, Waste receptacle, Reagent reservoirs, Refrigerator (2-8°C), freezer (-80°C ± 5°C & -20°C ± 5°C), Disposable gloves, Lab coat & sleeve protectors, Racks for cryo- & eppendorf tubes.

5.2. Reagents and Buffers preparation

Reagents	Buffer-type	Conc.	PH	Store	Preparation
NaH_2PO_4	Activation buffer	100mM	6.2	4°C	0.3g of NaH_2PO_4 in 25 mL H_2O
MES	Coupling/ Activation buffer	50mM	5.0	4°C	0.642g of MES in 60 mL H_2O
PBS-TBN	Wash/ storage buffer		7.4	4°C	5g BSA filtrated & 150 µL Tween20 & 0.05g sodium azide in 100mL PBS
PBS-CR			7.4	4°C	For 10mL: 5mL PBS + 5 mL CR
EDC	Coupling agent	50mg/mL		20°C	200µL H_2O into 10mg vial of EDS → prepare immediately before use!
Sulfo-NHS		50mg/mL		4°C	200µL H_2O into 10mg vial of Sulfo-NHS
PBS-5%BSA	Blocking/ storage buffer		7.4	4°C	5g BSA & 0.05 g/mL Sodium Azide in 100 ml PBS

Table 5: Preparation of the Reagents and Buffers.

6. Procedure

6.1. Covalent coupling of the Ags to the beads

Covalent coupling to carboxylated paramagnetic microspheres/beads (MagPlex microspheres, Luminex Corp., Austin, TX, USA) as described by Ambrosino et al., and the Luminex Corporation.
The final bead coupling is started from a microsphere concentration of $5x10^6$ beads and the testing phase is started from a microsphere concentration of $1x10^5$ beads.

1. Remove all reagents from the refrigerator to equilibrate to room temperature for 20-30 minutes.
2. Calculate the volumes needed for points 4, 11, 16,
3. Vortex (10-20 sec) and sonicate (10-20 sec) the 1mL stock microspheres (beads) (3x).
4. ***When taking $5x10^6$ beads***, starting from a microsphere stock concentration of $12.5x10^6$/1mL beads. Add 400µL of the beads in a 1.5mL low binding eppendorf (reaction tube).
   When taking $1x10^6$ beads, starting from a microsphere stock concentration of $12.5x10^5$ beads. Add 80µL of the beads in a 1.5mL low binding eppendorf (reaction tube).

$$\text{Volume of stock needed} = \frac{(\#\text{ of beads to couple})}{(\text{conc of stock vial})}$$

$$\text{Volume of stock needed} = \frac{(5 \cdot 10^6 \text{ beads})}{(12.5 \cdot 10^6 \text{ beads/mL})}$$

$$\text{Volume of stock needed} = 0.4\text{mL or } 400\ \mu\text{L}$$

5. Centrifuge at ≥ 8000rpm for 3 min.
6. Wash the microspheres 2x.

- Place the reaction tube into a magnetic separator for 5 min.
- When the beads stick to the side, remove the supernatant (SN) with a transfer pipette.
- Add 500µL NaH_2PO_4 (activation buffer)
- Vortex (10 sec) and sonicate (10 sec) to disperse the beads.

Magnetic seperator

7. Place the reaction tube back into the magnetic separator for 5 min and remove the SN with a transfer pipette.
8. Add 480µL 0.1 M NaH_2PO_4 (100 mM) into the reaction tube
9. Vortex and sonicate for 10-20 sec.
10. Vortex the provided Sulfo-NHS tube for 10 sec.
11. Add 10µL Sulfo-NHS to the reaction tube.
12. Immediately before use dissolve 10mg vial of EDC in 200µL of H_2O. Mix the EDC vial gentle and vortex for 10-20 sec. NOTE: EDS will degrade once exposed to the moisture in the atmosphere and the activation buffer. So, prepare all of the EDS prior to the next step and add quickly. *(Cf: 50 mg/mL → 10mg EDC/200µL dH_2O = 0.05mg/µL = 50mg/mL).*
    - Add 10µL EDC solution into the reaction tube.
13. Vortex the reaction tube for 10 sec.
14. Wrap the reaction tubes in tinfoil to protect the beads from light and incubate at room temperature for ±20 minutes on a rotator at 15-30rpm.
15. Centrifuge at ≥ 8000rpm for 3 min.
16. Wash the microspheres 3x as follows:
    - Place the reaction tube into a magnetic separator for 5 min.
    - When the beads stick to the side, remove the SN with a transfer pipette.
    - Add 500µL MES (Coupling/Activation buffer)
    - Vortex the reaction tube for 10 sec and sonicate for 10 sec to disperse the beads.
17. Place the reaction tube back into the magnetic separator for 5 min and remove the SN with a transfer pipette.
18. Calculate the volume of the Ag used in the reaction to obtain a desired concentration of 4µg/10^6 beads (see table 1 and 2 on the first page).
    - For example → Stock concentration = 2000µg/mL= 2µg/µL
    - So, add 10µl Ag and 400µl MES into the reaction tube

$$\text{Volume of Ag needed} = (\#\text{ of beads to couple}) * \left(\frac{\text{Desired Ag conc}}{\text{Stock Ag conc}}\right)$$

$$\text{Volume of Ag needed} = (5\text{x}10^6 \text{beads}) * \left(\frac{4\mu g}{2\mu g/\mu l}\right)$$

$$\text{Volume of Ag needed} = 10\mu l$$

19. Vortex (10 sec).
20. Wrap the reaction tubes in tinfoil to protect the beads from light and incubate at room temperature for ± 2.5 hours on a rotator at 15-30rpm.
21. Centrifuge at ≥ 8000rpm for 30 sec.

22. Place the reaction tube back into the magnetic separator for 5 min. When the beads stick to the side, remove the SN with a transfer pipette.
23. Add 500µL of PBS-TBN (Wash buffer) into the reaction tube.
24. Vortex (10 sec) and sonicate (10 sec).
25. Wrap the reaction tubes in tinfoil to protect the beads from light and incubate at room temperature for ± 30 min on a rotator (50rpm).
26. Centrifuge at ≥ 8000rpm for 30 sec.
27. Wash the microspheres 3x.
    - Place the reaction tube into a magnetic separator for 5 min.
    - When the beads stick to the side, remove the SN with a transfer pipette.
    - Add 500µL of PBS-TBN (Wash buffer). Vortex (10 sec) and sonicate (10 sec) to disperse the beads.
28. Place the reaction tube back into the magnetic separator for 5 min. When the beads stick to the side, remove the S with a transfer pipette.
29. ***When taking 5x10^6 beads:***
    Add 125µL of PBS-TBN (Wash buffer) and 125µL diluant CR (Charles River, MFIA: Multiplexed Fluorescence ImmunoAssay) into the reaction tube.
    When taking 1x10^6 beads:
    Add 25µL of PBS-TBN (Wash buffer) and 25µL diluant CR (Charles River, MFIA: Multiplexed Fluorescence ImmunoAssay) into the reaction tube.
30. Vortex (10 sec).
31. Repeat the covalent coupling of the Ags to the Beads for each Ag
32. Stock the reaction tubes in 4°C in the dark.

6.2. Counting on a Hemacytometer

Counting of the beads on a Hemacytometer is performed to check if there is a loss of beads. The final concentration of beads must be 20000 beads/µL. That means that the counted amount of a 1:10 dilution has to be 200 beads within one 4x4 corner grid.

33. Make a 1:10 dilution with dH_2O.
    - 90µL dH_2O.
    - 10µL stock solution
34. Count (in 4x4 corner grid) x dilution factor (10) x 10 = # beads/µL.

6.3. Bead-based Immunoassay

The immunoassay is described as by Khaireh et al., Ambrosino et al., and the Luminex Corporation.

1. Prepare all the reagents.
2. Resuspend the coupled beads by vortex (10sec) and sonication (10sec).

3. Prepare the coupled microspheres by diluting the microsphere stocks to a final concentration of 1000 beads/well (Vf= 25µL/well) in PBS and CR (Charles River) diluents.
   - Example for 1 whole plate →25µL/well x 100 = 2.5mL.
   - Cf: Final concentration: 1000 beads/well = 40 beads/µL
   - Vf: Final volume: 25µL/well → 2500 µL/plate.
   - Ci: Counted Bead concentration (Beads/µL).
   - $\text{Volume of beads needed (Vi, in µL)} = \left(\frac{Cf \times Vf}{Ci} \right)$

   Add 25ul (1000 beads) or 50µL (2000 beads) of each bead-set in the appropriate wells. Pipette up & down 5x.
4. Put the plate on the magnetic separator for 2 min and cover them with tinfoil to protect them from the light.
5. Add 50µL of the diluted serum (e.g. 1:100 or 1:200) in the appropriate wells (as described in table 4)(vortex and sonicate 10 sec before adding). Pipette up & down 5x.
6. Cover the plates with sealing tape and tinfoil to protect the beads against light
7. Incubate for 1 hour on a shaker (600rpm)
8. Place the plate on the magnetic separator for 5 min and remove the SN. Wash the plates with 200µL of wash solution (PBS-TBN) and remove the SN with a magnetic separator (Pipette up & down 5x).
9. Repeat step 10 for a total of 2 washes
10. Place the plate on the magnetic separater for 5 min and remove the SN. Add 100µL of secondary Ab (R-phycoerythrin F $(ab')_2$ 1:500 (1µg/mL) dilution in PBS-TBN) in each well (Pipette up & down 5x).
11. Cover the plates with sealing tape and tinfoil to protect the beads against light
12. Incubate for 45 min on a shaker (600rpm)
13. Place the plate on the magnetic separator for 5 min and remove the SN. Wash the plates with 200µL of wash solution (PBS-TBN) and remove the SN with a magnetic separator (Pipette up & down 5x).
14. Repeat step 14 for a total of 2 washes.
15. Place the plate on the magnetic separator for 5 min and remove the SN. Resuspend the beads in 100µL of PBS-TBN (5min rotate the plate) Pipette up & down 5x.
    → NOTE: if the plate is read the next day, cover is with sealing tape and tinfoil. The next day shake the plate for 10 min before reading.
16. Read the plate with the MAGPIX (luminex) (50µL or 25µL for 400 beads)

6.4. MAGPIX plate reading

The MAGPIX is read as described by the Luminex Corporation.

- **Starting xPONENT**
  Perform the following steps to launch xPONENT:
  - On the PC desktop, click the Luminex xPONENT icon, or click **Start** > **All Programs** >**Luminex** > **xPONENT** > **Luminex xPONENT**.
  - Login on the **System login** tab, type your used ID

- **Initial Startup**
  When you turn on the system for the first time, perform the following procedures:
  1. Adjusting the Sample Probe Height
  2. Revive After Storage (Luminex) Routine
  3. Calibration/Verification

- **Adjusting the Sample Probe Height**
  Adjust the sample probe height to ensure that the probe drops far enough into the well to acquire sample.

  NOTE: Ensure that there is no liquid in the wells or reservoirs before adjusting the sample probe height.

  1. On the **Home** page, click **Probe and Heater** under **Daily Activities**. The **Probe & Heater** tab opens.
  2. Use well D6 (this is the center of a standard 96-well plate).
  3. Ensure that the well location is selected on the plate image. A green pin marks the selected well.
  4. Based on the type of plate you are using, place alignment disks or an alignment sphere in the well.
     - For a standard 96-well plate - none
     - For a Filter-bottom plate - two 5.08 mm disks
     - For a Mylar-bottom plate - two 5.08 mm disks
     - For a conical (v-shaped) plate - one sphere
  5. Click **Eject** to eject the plate carrier.
  6. Place the off plate reagent block on the plate carrier. Make sure it is well seated so that it clips into place.
  7. Place a strip well (provided with the Calibration and the Performance Verification kit) in the off-plate reagent block.
  8. In the **Strip Wells** section, click **SD1**.
  9. Verify that the reservoir is empty.
  10. In the **Reservoir** section, click well **RB1**.
  11. Verify that the plate is not warped. Warped plates can lead to incorrect probe height adjustment.
  12. Place the plate on the plate carrier with well A1 positioned as indicated on the plate carrier.
  13. Click **Retract** to retract the plate carrier.

14. Type a name for the plate in the **Plate Name** box.
15. Click **Auto Adjust Height**. The probe automatically adjusts itself to the locations you selected.
16. Click **Eject** to eject the plate holder. If you used alignment disks or spheres, remove them from the plate.

NOTE: When you adjust and save the probe height settings for all three areas under a plate name, all areas retain the adjustment.

WARNING: Correct sample probe height is critical to successful sample acquisition and calibration. Problems with the sample probe can lead to fluid leaks and inhibit sample acquisition.

CAUTION: Ensure that the probe height is set correctly before calibrating

Figure 3: Sample Probe Height Adjustment

- **Revive After Storage Routine**

NOTE: The **Revive After Storage** routine is necessary when the system runs for the first time and is recommended when the system has been idle for more than a week.
After you have adjusted the sample probe height, run the **Revive After storage (Luminex)** routine.

1. Open the **Maintenance** page, then the **Cmds & Routines** tab.
2. Select **Revive After Storage (Luminex)** from the drop-down list. The **Revive After Storage** routine performs the following commands:
   - **Prime**

- **Rinse**
- **Alcohol Flush**
- **Rinse**

3. Add 70% isopropanol or ethanol to reservoir RB1 on the off-plate reagent block as indicated on the **Cmds & Routines** tab.
   NOTE: The rinse reservoir (RD1) should be empty.
4. Click **Retract.**
5. Click **Run.**
   After the **Revive After Storage** routine is complete, run the **System Initialization** routine.

- **Calibration - Verification**
  Calibration normalizes the settings for the system and ensures optimal and consistent microsphere classification. Verification uses system controls to ensure that the analyzer is functioning properly with current calibration settings.

1. On the **Home** page, click **System Initialization** under **Daily Activities**. The **Auto Maint** tab opens.
2. Click the **Calibration/Verification** option under **Automated Maintenance Options** section.
3. Import the **Calibration Kit** lot information from the CD provided with the kit or select the appropriate kit from the drop down menu if the kit information has been preloaded.
   NOTE: See the *Adding or Importing CAL and VER Kit Information* section on how to import the kit.
4. Import the **Performance Verification Kit** lot information from the CD provided with the kit or select the appropriate kit from the drop down menu if the kit information has been preloaded.
   NOTE: See the *Adding or Importing CAL and VER Kit Information* section on how to import the kit.
5. Vortex the xMAP calibrator, verification, and fluidics containers at a medium speed for approximately 10 seconds to ensure homogeneity. Do not dilute xMAP calibrator, verification, or fluidics agents.
6. Click **Eject** on the status bar.
   NOTE: To ensure that you get the necessary bead count, invert the calibrator and verifier vials perpendicular to the strip well as you add drops to the wells. This ensures that the maximum fluid drop size is dispensed into the wells.
7. Luminex recommends adding 6 drops of each reagent into the designated well.
8. Verify that reservoir RB1 is 3/4 filled with 70% isopropanol or ethanol.
   NOTE: The rinse reservoir (RD1) should be empty.
9. Click **Retract.**
10. Click **Run**.

- **Protocol Procedures**

Creating an Allele Call Protocol

Protocols > Protocols > Create New Protocol

This protocol contains no standards or controls. An allele call protocol compares groups of two, three, or four analytes to identify genotypes.

To create an allele call protocol:

1. Open the **Protocols** page, then open the **Protocols** tab. Click **Create New Protocol**.
   The **Protocol Settings** tab opens.
2. In the **Name** box, type the name of the protocol.
3. In the **Description** box, type a description of the protocol.
4. In the **Version** box, type the version of the protocol.
5. In the **Manufacturer** box, type the manufacturer information for the protocol.
6. Define the settings in the **Acquisition Settings** section.
   NOTE: Final washes are required for proper analysis. If you are not performing a final wash step on your plate before acquisition on the MAGPIX instrument, enable **Sample Wash** here. This automatically washes each sample.
7. Define settings in the **Analysis Settings** section, selecting **Allele Call** as the analysis type.
8. Click **Next**. The **Analytes** tab opens.
9. Click the desired analyte (bead ID) in the numbered grid. Information about the analytes is displayed in a list on the right side of the grid. For an allele call analysis, you must select a group of two, three, or four analytes.
10. Click **Group** to group analytes for the allele call. The grouped analytes display in a list to the right. Select more analytes and then click **Group** again if you want to add another group to the analysis.
11. Click and type an analyte name in the **Name** column to the right of the analyte grid.
12. In the **Count** box, type the desired bead count for each analyte. Click **Apply All**.
13. To set a bead count and the units for a single analyte, click in the **Units** and **Count** columns directly to the right of the analyte grid, and type a units value and bead count.
14. Click in the **Call %** column and type a value to set an individual analyte's call percentage.
15. Click **Next**. The **Plate Layout** tab opens.
    - To add well commands, select the appropriate wells and mark them as unknown, standard, control, background, or wash. You can also delete commands that you've added and

change the starting location on the plate. If you want to run in replicate, change the **Replicate Count** to the appropriate number and the **Grouping** to your preferred grouping method.

- Delete a command by clicking the well, and clicking **Delete**. The **Delete Options** dialog box opens. Select **Delete** just the selected wells to delete a single well command, or **Delete** all wells containing these samples to delete all wells with the same command.

NOTE: The **Post Batch** routine runs after the batch is complete. This routine is selected by default when the protocol or batch is set up. The default **Post Batch** routine can be changed in the **Batch Options** tab of the **Admin** page.

NOTE: For more information about

- **Using the Batches Page**
  1. Open the **Batches** page.
  2. Click one of the following:
     - **Create a New Batch from an Existing Protocol**
     - **Create a New Batch from a new Protocol** - if you select this option, you can save the protocol and/or stds/ctrls information.
     - **Create a New Multi-Batch**
  3. Type the batch name in the **Batch Name** box.
  4. Type an optional description of the batch in the **Enter Optional Description** box.
  5. If you are creating a batch from an existing protocol, select the protocol in the list. Click **Next**. If the protocol uses standards and/or controls, the **Stds & Ctrls** tab displays.
  6. If you selected **Create a New Batch from a new Protocol**, the **Stds & Ctrls** tab displays.
  7. The **Plate Layout** tab appears. View the details of the active reagents, apply different assay standards/controls, or manually enter new information. Click **Next.**
  8. On the **Plate Layout** tab, assign well commands for this batch.
  9. Click **Run Batch** to begin batch acquisition, or click **Save** to save batch information to the **Pending Batch** list to be run at a later time.

     NOTE: If the batch spans more than one plate, the tray ejects automatically when all defined wells have been

acquired. A dialog box displays prompting you to insert the next plate.

- **Create a New Batch from an existing Protocol**
  Read the instructions provided with the assay kit you are using.
  1. Open the **Batches** page.
  2. Click **Create a New Batch from an existing Protocol**.
  3. Type the batch name in the **Batch Name** box.
  4. Type a description about the batch in the **Enter Optional Description** box.
  5. Click a protocol you wish to use in the **Select a Protocol** list.
  6. Click **Next**. If the protocol uses standards, controls, or both, the next tab that opens is the **Stds & Ctrls** tab. View the details of the active reagents or apply different assay standards, controls, or both or manually enter new information. Select **Next**. If the selected protocol does not use standards, controls, or both, the next tab that opens is the **Plate Layout** tab.
  7. On the **Plate Layout** tab, assign well commands for this batch. See *Plate Layout Tab* for a complete description of the commands and options on this tab.
  8. Click **Run Batch** to begin batch acquisition, or click **Save** to save batch information to the **Pending Batch** list to be run at a later time.

     NOTE: If the batch spans more than one plate, the tray ejects automatically when all defined wells have been acquired. A dialog box opens, prompting you to insert the next plate.

- **Create a New Batch from a New Protocol**
  Click this option to create a new batch from a new protocol. This option enables you to create a protocol while you are creating the batch.
  1. Open the **Batches** page.
  2. Click **Create a New Batch from a new protocol** to open the **Settings** tab.
  3. Type a name in the **Name** box.
  4. Type a description in the **Description** box.
  5. Define the settings in the **Acquisition Settings** section. These are **Volume**, **XY Heater** (enable/disable and set temperature), **Plate Name**, and enable/disable **Sample Wash**.

     NOTE: Final washes are required for proper analysis. If you are not performing a final wash step on your plate before acquisition on the MAGPIX instrument, enable **Sample Wash** here. This automatically washes each sample.

If you select **Qualitative**, **Quantitative**, or **Allele Call** from the **Analysis Type** list, you can select **Analyze results while acquiring samples** to view real-time analysis.

6. Next, complete the Analysis Settings. These include **Analysis Type**, **MFI** enable/disable (used for Allele Call analysis), # of standards, # of controls, either **Fit all Standards** or **Mean of Replicates**.
7. Click **Next**. The **Analytes** tab opens. Click the analytes of interest in the numbered grid. Information about the analytes opens in a list on the right side of the grid. Name the analytes.
8. To change the **Default Analysis**, click **Change**. The **Analysis Settings** dialog box opens.
9. Select the analysis method from the **Method** list. Click **OK** to change the default analysis for analytes to be selected. Click **Apply to All Analytes** to apply the selection to all analytes. The **Analysis** dialog box closes.
10. Type a unit of measurement in the **Units** box.
11. Type the desired bead count for each analyte in the **Count** box.
12. If you click **Apply All**, this applies to all analytes.
13. To change individual units or counts, change them in the analyte table.
14. Click **Next**. The **Stds & Ctrls** tab opens if you selected an analysis type other than **None**.
    - If you are using an assay standard/control kit, click **Apply Std/Ctrl Kit**. In the **Select Std/Ctrl Kit** dialog box, click the kit from the list and click **OK**. Applying a kit only works for kits already installed, but you can also manually type the information.
    - If you are not using a kit, type the appropriate information in the **Standard Information** and **Control Information** sections. The number of standards and/or controls in these sections is defined on the **Settings** tab in the **Analysis Settings** section. If your batch uses controls, enter the appropriate values for **Expected Values**. Click **Low Value** from the **Show** list, and enter the low value for each analyte. Click **High Value** from the **Show** list, and enter the high value for each analyte. Reagent information is not required for a custom batch unless you want to use the analysis feature.
15. Click **Next**. The **Plate Layout** tab opens.
    - To add well commands, click the appropriate wells and mark them as unknown, standard, control, background, or wash. You can also delete commands that you've added, and change the starting location on the plate. If you want to run in replicate, change the **Replicate Count** to the appropriate

number and the **Grouping** to your preferred grouping method.

- As you add commands to your plate, they are listed in the **Command Sequence** list. Here you can give each of your wells an ID. You can also import an ID list and move your commands up and down to change the plate location.

16. Click **Single Step** to acquire the first well, then pause acquisition.
17. Click **Run Batch** to start acquisition, or **Save** to save the batch for a later time. You can also save the protocol and/or standard and control information by clicking **Save Prtcl**.

NOTE: If the batch spans more than one plate, the tray ejects automatically when all defined wells have been acquired. A dialog box displays prompting you to insert the next plate.

- **Exporting a Batch**
  1. Open the **Batches** page.
  2. In the **Pending Batches** section, click the batch you want to export, then click **Export**.
     The **Export Batch** dialog box opens.
     NOTE: You can export batches, but not multi-batches.
  3. Click **Browse**. The **Select File** dialog box opens.
  4. Navigate to the location to which you want to save the file, then click **Save**.
  5. Click **OK** in the **Export Batch** dialog box.
     NOTE: When exporting a large batch and including the LXB files, the export process may take ten minutes or more.

- **Editing a Batch**
  1. Open the **Batches** page.
  2. Click the batch you want to edit, then click **Edit**. The **Protocol** tab opens.
  3. Edit the information as needed on the **Protocol**, **Std & Ctrls**, and **Plate Layout** tabs. For the tab, confirm that the plate layout conforms to your specific assay instructions.
  4. Click **Save** on the **Plate Layout** tab.
     NOTE: Batches saved to a multi-batch cannot be edited or deleted unless they are removed from the multi-batch. However, you can edit themulti-batch itself. To remove a batch from a multi- batch, click on a well in the plate layout, and click **Remove**.

- **Daily Activities**
  - **System Initialization** - Perform a system initialization routine.

NOTE: Luminex recommends weekly calibration and daily verification. For daily use, verify your System Initialization setting is set to Fluidics Prep and Performance Verification in the Admin System Setup tab.

Refer to *Maintenance Page* for detailed maintenance instructions.

- **Shutdown** - Perform the shutdown routine.
- **Probe and Heater** - Adjust the probe height or plate heater.
- **Drive Fluid Lot** - Enter the Drive Fluid lot number, which is printed on the box in which the fluid container was shipped. This information is optional.
- Create a **New** Batch **from a new Protocol** - Creates a new batch from a new protocol by opening the **Settings** tab of the **Batches** page. You can create protocols while creating a batch, and can save the protocol before or after you run the batch.
- Create a **New** Batch **from the highlighted Protocol below** - Creates a new batch using a selected protocol from the **Installed Protocols** list. This button displays the same fields as the **Create a new batch from existing Protocol** button on the **Batches** page.
- **Scroll** - Use the up and down arrows to scroll through the list of installed protocols.
- **View** - Opens the **Settings** tab of the **Protocols** page to view the selected protocol. This tab enables viewing the settings, analytes, and plate layout for the selected protocol.
- **Sys Info** - Opens the **System Info** tab of the **Maintenance** page. If the instrument is connected and powered on, the **System Information** page displays licensing information, the instrument serial number, the date of the last Calibration, Verification, Fluidics tests, and other important information.
- **Reports** - Opens the **Reports** tab of the **Results** page.
- Return to the **Home** page at any time by clicking **Home** at the top of the screen.

- **Shutting Down the Analyzer**

Run the daily shutdown routine to prevent clogs and crystallization of salt in the sample probe. Clogs and crystallization of salt in the sample probe can cause problems with calibration, verification, and data acquistion; they can also cause sample splashing. Shut down the system properly to ensure system integrity.

1. On the **Home** page, click **Shutdown**. The **Auto Maint** tab opens, with **System Shutdown** selected.
2. Click **Eject**.
3. Fill reservoir **RA1** with 3/4 of DI water.

4. Fill reservoir **RC1** with 3/4 of 10%-20% household bleach solution.
5. Verify that reservoir **RD1** is empty.
6. Click **Retract**.
7. Click **Run**.

- **Logging Off and Exiting**
  To log off and exit xPONENT:
  1. Click **Logoff** at the top of the page.
  2. When the **Confirm** dialog box opens, click **OK**. This opens the **Log In** page, with **Exit** on the left tab.
  3. Click **Exit** to exit the application.

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ANNEX

Plate setup testing phase

		Positive Serum dilution								Neg serum	blanco	
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600			
1:250		Strongest sera											GLURP coated beads
1:500		Strongest sera											
1:250		Strongest sera											BSA coated beads
1:500		Strongest sera											

Plate1: testing the strongest sera per Ag in monoplex

		Positive Serum dilution								Neg serum	blanco	
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600			
Sec. Ab	1:500	Pool sera											Ag 1
	1:500	Pool sera											Ag 2
	1:500	Pool sera											Ag 3
	1:500	Pool sera											Ag 4
	1:500	Pool sera											Ag 5
	1:500	Pool sera											BSA
	1:500	Pool sera											Microsphere working mixture
	1:500	Pool sera											

Plate2: testing the pool of positive serum per Ag in monoplex and multiplex

		Positive Serum dilution						Neg serum	blanco	
		1:100	1:100	1:400	1:400	1:1600	1:1600			
Sec. Ab	1:500	Pool sera								Microsphere working mixture
		Pool sera								

Plate3: testing the pool of positive serum in multiplex

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Plate setup final Bead-based Immunoassay

Secondary Antibody dilution 1:500

A	Sera sample 1	Sera sample 9	Sera sample 17	Sera sample 25	Sera sample 33	Sera sample 41	Sera sample 49	Sera sample 57	Sera sample 65	Sera sample 73	High positive control	High positive control	Microsphere working mixture (1000 beads/25µl)
B	Sera sample 2	Sera sample 10	Sera sample 18	Sera sample 26	Sera sample 34	Sera sample 42	Sera sample 50	Sera sample 58	Sera sample 66	Sera sample 74	Medium positive control	Medium positive control	
C	Sera sample 3	Sera sample 11	Sera sample 19	Sera sample 27	Sera sample 35	Sera sample 43	Sera sample 51	Sera sample 59	Sera sample 67	Sera sample 75	Low positive control	Low positive control	
D	Sera sample 4	Sera sample 12	Sera sample 20	Sera sample 28	Sera sample 36	Sera sample 44	Sera sample 52	Sera sample 60	Sera sample 68	Sera sample 76	Negative control	Negative control	
E	Sera sample 5	Sera sample 13	Sera sample 21	Sera sample 29	Sera sample 37	Sera sample 45	Sera sample 53	Sera sample 61	Sera sample 69	Sera sample 77	Blanco control	Blanco control	
F	Sera sample 6	Sera sample 14	Sera sample 22	Sera sample 30	Sera sample 38	Sera sample 46	Sera sample 54	Sera sample 62	Sera sample 70	Sera sample 78			
G	Sera sample 7	Sera sample 15	Sera sample 23	Sera sample 31	Sera sample 39	Sera sample 47	Sera sample 55	Sera sample 63	Sera sample 71	Sera sample 79			
H	Sera sample 8	Sera sample 16	Sera sample 24	Sera sample 32	Sera sample 40	Sera sample 48	Sera sample 56	Sera sample 64	Sera sample 72	Sera sample 80			

8.3 ANNEX 3 (ELISA results positive control selection)

Serum ID	CSP	GLURP	GLURP P3	LSA1-41	LSA1-J	LSA3-NR2	LSA3-RE	PfGLURP R2	PfMSP1-19	SALSA1	SALSA2	SR11.1	STARP-R	SALIV2	PvMSP1-19	PV-like CSP	PvVK210-CSP	PvDBP	Pf13	PmCSP
5035	36.38	84.24	1.23	88.67	66.99	46.34	40.31	42.23	108.62	33.86	18.11	29.74	4.41	4.68	10.41	0.63	1.39	8.11	39.33	
5051	20.04	104.89	12.94	111.05	106.29	8.20	22.28	74.13	76.46	38.71	8.50	33.04	4.31	2.88	9.63	6.30	3.31	16.17	5.26	
5191	8.39	100.94	0.72	63.99	54.12	1.55	65.60	54.66	31.28	9.15	0.61	80.51	12.96	2.17	4.68	0.27	0.89	7.08	6.01	
5208	56.89	66.87	0.65	76.40	21.45	1.65	19.99	88.76	111.40	0.46	2.33	17.52	3.76	2.35	9.14	0.12	0.00	32.68	3.54	
5248	21.85	33.83	1.21	11.44	2.35	7.59	99.90	37.18	94.46	2.45	18.21	12.02	10.83	3.36	80.15	1.02	1.29	39.77	44.15	
5286	0.82	40.91	-1.78	100.09	94.54	89.47	19.21	63.74	105.43	-0.76	1.00	5.71	2.68	7.05	10.12	1.45	0.36	19.69	6.86	
5373	7.28	18.65	0.29	9.42	2.25	1.93	2.22	39.13	109.61	2.15	38.65	16.82	12.26	3.92	3.76	0.29	0.44	24.72	15.09	
5333	0.46	93.52	48.29	24.06	28.05	112.33	23.90	57.24	106.99	3.76	63.61	13.47	-1.06	38.67	24.61	-1.43	0.67	33.90	11.17	
C1 0006	0.31	0.02	0.00	2.12	2.12	0.19	1.55	4.38	0.00	0.08	1.11	1.79	0.08	9.80	105.84	6.04	12.20	33.22	7.31	
C1 0026	-0.10	-0.94	0.31	-0.21	-0.08	-1.63	-0.06	-0.04	0.78	-0.60	-0.08	15.14	-3.27	4.08	112.47	3.90	4.54	80.86	0.67	
C1 0059	1.78	4.25	1.52	3.95	3.64	1.84	2.91	5.34	2.05	0.57	0.51	8.03	-0.78	10.96	75.88	4.89	23.92	30.04	2.89	
C1 0082 3215/001 3/04	16.36	1.74	1.46	14.15	2.29	3.14	52.64	25.34	4.56	65.30	10.37	106.61	-0.32	7.11	121.02	8.42	12.03	16.61	3.39	50.48
Pool sera %	44.9	139.0	34.0	121.7	112.1	29.3	135.5	96.4	144.7	102.8	45.8	105.2	4.5	4.1	110.2	-0.1	-1.7	73.1	23.7	
Pool sera average COD	1.112	2.61	0.64	2.29	2.11	0.55	2.55	1.81	2.72	1.93	0.86	2.609	0.112	0.10	2.732	0.0015	-0.0425	1.812	0.588	
Neg COD	-0.049	-0.053	-0.025	-0.088	-0.039	-0.112	-0.054	-0.147	0.018	-0.079	-0.073	-0.056	0.019	0.041	0.195	-0.046	-0.037	0.059	0.025	
Blanco COD	0.08	0	0.002	-0.003	-0.001	-0.003	0.009	-0.003	0.001	-0.002	-0.001	-0.001	0	-0.001	0.160	-0.004	0.001	0.003	0.003	
Ratio absolute value	22.70	49.29	25.58	25.99	54.03	4.91	47.51	12.33	151.06	24.46	11.78	46.69	5.92	2.49	14.01	0.03	1.15	30.71		

strong+++

weak+

pooled sera

8.4 ANNEX 4 (MFI-values of the positive serum control per Ag per dilution)

Antigens	1:100 (1000 beads)	1:400 (1000 beads)	1:1600 (1000 beads)	1:100 (2000 beads)	1:400 (2000 beads)	1:1600 (2000 beads)
GLURP	31553	27657.5	14327	27647.5	25076.5	13140
GLURP	29125.5	26416.5	14560	26284	24051.5	11810
GLURP	30895	27223.5	15640.5	27079	25181	
CSP	9699	3171.5	1187	8380.5	2579.5	874
CSP	9187	3126	1162.5	7731	2504.5	846.5
CSP	9384.5	3292.5	1113.5	8224	2643.5	
GLURP-P3	24359.5	15231.5	7136	22147.5	13366.5	5677
GLURP-P3	24053.5	15500.5	7011	21364	12449.5	5185.5
GLURP-P3	23654	15605.5	7088	22826	13525.5	
LSA1-41	33120	30112.5	26393.5	28824.5	29305.5	24079
LSA1-41	32670.5	30945.5	26524	27611	27277.5	21978
LSA1-41	32521.5	30724.5	25424.5	28780	28412.5	
LSA1-J	31726	27656	18008	25470.5	25736.5	15250.5
LSA1-J	30610.5	27982	17454.5	24263	24741.5	13913
LSA1-J	31483	27871.5	18163.5	26746	25400	
LSA3-NR2	18556.5	8594	2782.5	17096.5	6762.5	2316
LSA3-NR2	18662	8281.5	2935	15943	6725.5	2139
LSA3-NR2	18680.5	8458	3044	17126	6755	
LSA3-RE	32661.5	30209	19802	28667.5	28189	16718
LSA3-RE	31345.5	29253.5	19600.5	27248	26852.5	15433.5
LSA3-RE	32580	29937	19442.5	29489	27715	
Pf GLURP R2	44785	41296	22327.5	44570.5	34331.5	19074.5
Pf GLURP R2	45163.5	39357.5	24323.5	41118.5	33555.5	16316.5
Pf GLURP R2	44654	41337.5	22171.5	47249	33947	
Pf MSP1-19	34768	32732	30952	32922.5	30835.5	29481
Pf MSP1-19	33850.5	32404.5	30771.5	32803	30430.5	27536.5
Pf MSP1-19	34858.5	32893	28346.5	33636	30750.5	
SALSA1	3082	1138	309	2368	889.5	349
SALSA1	2519.5	940.5	343.5	2244	899	304.5
SALSA1	2741.5	1074.5	424.5	2205	953	
SALSA2	20497	8562.5	2907.5	17650.5	7356	2613.5
SALSA2	18862	8031	3009	17204.5	6763.5	2386.5
SALSA2	19308.5	8628.5	3266.5	17228	7068	
SR11.1	1506	775	267	1240	770	360.5
SR11.1	1365.5	714.5	343.5	1178	681.5	303.5
SR11.1	1248	698.5	354.5	1129	570	
STARP-R	11737	3894.5	1128.5	9480	2834.5	894.5
STARP-R	11065	3370.5	1166.5	9277	2720	830.5
STARP-R	10784	3764	1188	8969	2959.5	
SALIV2	3019	468	287	1120.5	827.5	183
SALIV2	1313.5	530.5	241	1521	575	207.5
SALIV2	1084	475	137.5	1763.5	507	
Pv MSP1-19	7785	6788	6253	7370.5	6874.5	5858
Pv MSP1-19	7083.5	6627	6080.5	7080	6201.5	5560.5
Pv MSP1-19	7297	6597.5	5853.5	7427	6649.5	
Pm CSP	738	161.5	21	583.5	104	18
Pm CSP	660	117.5	18.5	529	81.5	12.5
Pm CSP	645	143.5	22	519	98.5	
Pv like CSP	559	210	74	461	164.5	63
Pv like CSP	439.5	171.5	85.5	416.5	146	56.5
Pv like CSP	434	195.5	73	402	177.5	
PvVK210 CSP	227	128	43	192.5	83	30
PvVK210 CSP	170.5	75	51.5	168	63	32.5
PvVK210 CSP	151	107.5	42.5	177	85	
Pf13	6821	2075.5	561	5623.5	1634.5	521
Pf13	6210.5	1948.5	578.5	5641.5	1540.5	469.5
Pf13	6305	2054.5	654.5	5634	1687	
Pv DBP	29935.5	19441	10250.5	28038.5	16934.5	8673
Pv DBP	28809.5	18925.5	9979.5	26751	16469.5	7952.5
Pv DBP	29869.5	19182.5	9971.5	28157	16968.5	