Development of quantitative suspension array assays for six immunoglobulin isotypes and subclasses to multiple *Plasmodium falciparum* antigens

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Key words

IgG, subclasses, IgM, IgE, multiplexed antigens, *Plasmodium falciparum*.

ABSTRACT

Background: Quantitative suspension arrays are powerful immunoassays to measure antibodies against multiple antigens in large numbers of samples in a short time and using few microliters. To identify antigen targets of immunity for vaccine development against complex microbes like malaria, such technology allows the characterization of the magnitude and antigenic specificity of Ig isotypes and subclasses that are important for functional responses. However, standardized assays are not widely available.

Methods: We developed six quantitative suspension array assays for IgG1, IgG2, IgG3, IgG4, IgM and IgE specific to multiple *P. falciparum* antigens. Among commercially available sources, secondary and tertiary antibodies, as well as human purified antibodies for standard curves, were tested. Positive and negative controls included plasmas from malaria hyper-immune African adults and from malaria-naïve European adults, respectively. Reagents were selected and optimal antibody and test sample dilutions established according to sensitivity, specificity and performance of the standard curves. The variability between replicates and plates was assessed with 30 test samples and controls.

Results: Assays were able to detect *P. falciparum* antigen-specific antibodies for all isotypes and subclasses in samples from malaria-exposed individuals, with low background signal in blank wells. Levels detected in malaria-naïve individuals were overall low except for IgM. For the IgG2 and IgE assays, a triple sandwich was required for sensitivityStandard curves with 5-parameter logistic fit were successfully obtained in all assays. The coefficients of variation for measurements performed in different days were all < 30%, and < 5% when comparing duplicates from the same plate.

Conclusion: The isotype/subclass assays developed here were sensitive, specific, reproducible and of adequate quantification dynamic range. They allow performing detailed immuno-profiling to large panels of *P. falciparum* antigens to address naturally- and vaccine-induced Ig responses and elucidate correlates of malaria protection, and could also be applied to other antigen panels.

1. Introduction

In the assessment of humoral immunity against complex infections such as *Plasmodium* parasites, still affecting 95 countries and with 3.2 billion people at risk in 2015[1], it is key to have immunoassays that can reliably measure multiple immunoglobulins (Ig) and antigens in a mid-high throughput miniaturized manner. Antigen and isotype/subclass targets of naturally-acquired immunity[2, 3] need to be identified to characterize mechanisms of protection and find ways to induce them through vaccination.

Most malaria sero-epidemiological and vaccine studies only measure antigen-specific IgG[4, 5], since in the 60's it was established that transfer of purified IgG can control *P. falciparum* infection[6]. Nevertheless, antigenic targets of protection are unknown, and diverse Ig isotypes and subclasses are generated in response to malaria infection[7-10]. These various isotypes could be differentially elicited by antigens and have different effector functions, some of them being protective while others not[11-13]. It is generally known that IgG1 and IgG3, both considered cytophilic antibodies, are the main subclasses generated against *P. falciparum* antigens[14-16], but their relevance and function needs to be better studied. The most accepted mechanism by which IgG1 and IgG3 may protect against *P. falciparum* infection is through their ability to fix complement and mediate opsonizing phagocytosis[17, 18]. However, it needs to be better established whether non-cytophilic IgG2 and IgG4 antibodies, despite being present at low levels in exposed individuals, could be induced in detriment of cytophilic subclasses considered as protective, and to what extent their increase could be associated with risk of malaria. Furthermore, the role of IgM and IgE in malaria immunity has been less studied and merits more attention according to recent data associating those responses to protection[19] or risk[20-22], respectively. Therefore, an appropriate understanding of the magnitude and antigenic specificity of each of the Ig isotypes and subclasses is very important for the development of a new generation of effective vaccines.

Traditionally, the measurement of specific antibodies has been done by the enzyme-linked immunosorbent assay (ELISA)[23-25]. Although this classical technique has been very useful over the years, it demands significant amount of time, the use of relatively large sample

volumes and, importantly, only allows quantifying antibodies against a single antigen at a time. A mid-high throughput multiplex alternative technique is the quantitative suspension array technology (qSAT), particularly suited for large parasites like *P. falciparum* that is estimated to contain around 5,000 proteins, many of which are polymorphic and/or variant, and stage-specific. qSAT has several advantages compared to ELISA already demonstrated in many studies in diverse research areas[26-29]. For example, qSAT allows working with 5 or less microliters of plasma, serum or saliva, and simultaneously quantify up to 500 different proteins/antibodies, peptides, RNA or DNA fragments in a single well. In addition the qSAT is a very flexible platform that allows different antibody sandwiches, representing a perfect tool to assess the levels of different Ig isotypes and subclasses in large numbers of samples.

In this study, we have developed 6 different qSAT assays to measure antigen-specific IgG subclasses (1 to 4), IgM, and IgE using several panels of minimum 6 to 10 *P. falciparum* antigens. For this purpose, several antibody sandwiches were tested to choose the optimal combination for each isotype/subclass assay. In addition, isotype/subclass specific singleplex standard curves were developed to select sample dilutions for data analysis and to calibrate the assay. The variability of the assays between replicates and plates was also evaluated.

2. Material and methods

2.1. Human samples

A plasma pool made of 22 samples from malaria hyper-immune adults from Manhiça, Mozambique[30], was used as positive control. Fourteen individual plasma samples from European adults never exposed to malaria were used as negative controls. Test samples from 30 malaria-exposed individuals, adults and children, collected in the context of different immunological studies[31-33], were assayed in the setting up and assessment of the different assays.

Written informed consent was obtained from participants before sample collection; in the case of children the informed consent was obtained from parents or guardians.

Approval for the protocols was obtained from the Hospital Clínic of Barcelona Ethics Review Committee and the National Mozambican Ethics Review Committee.

2.2. P. falciparum recombinant antigens

A primary multiplex panel including 10 recombinant proteins with a broad range of immunogenicities was initially established to set up the IgG_{1-4} and IgM assays using the Luminex xMAP™ technology (Luminex Corp., Austin, Texas)[32]. The antigens were selected based on their important role as candidate vaccines, and for being representative of the different phases of the parasite life cycle. The panel included 4 pre-erythrocytic antigens: cell-traversal protein for ookinetes and sporozoites (CelTOS)[34], liver-stage antigen 1 (LSA-1)[35], sporozoite surface protein 2 (SSP2, also known as TRAP)[36] and circumsporozoite surface protein (CSP)[37]; and 6 erythrocytic antigens: apical membrane antigen 1 (AMA-1) from 3D7 and FVO strains[38-40], merozoite surface protein 1 ($MSP\text{-}1_{42}$) from 3D7 and FVO strains[41, 42], fragment II of region II of the 175 kDa erythrocyte binding protein (EBA-175 or PfF2)[43], and Duffy binding-like alpha (DBL-α) domain of *Pf*EMP-1[44]. *P. falciparum* AMA- and MSP-1 are polymorphic proteins, and the two most studied strains are 3D7 and FVO. Antigens based on primary sequences from both strains have been developed as vaccine candidates because of the strain-specific nature of antibody responses to many malarial antigens. Experimental vaccines based on only one genotype of these proteins have been tested in field trials showing different degree of protection depending on the circulating strain[45, 46]. As antibody responses to polymorphic proteins may vary in different populations, we included AMA-1 and MSP-1 from both strains in the panel to have a broader repertoire and check whether they elicited different IgM and IgG subclass responses. A bovine serine albumin (BSA)-coupled bead was also included in the multiplex for background determination. The pre-erythrocytic antigens were expressed in *Pichia Pastoris* and provided by Protein Potential, LLC (Rockville, Maryland, USA). AMA-1 3D7, EBA-175 and DBLα were provided by the International Centre for Genetic Engineering and Biotechnology (ICGEB).

AMA-1 FVO and MSP-1_{42} 3D7 and FVO were provided by the Walter Reed Army Institute of Research (WRAIR). The BSA was purchased to Sigma-Aldrich.

A secondary panel was used to set up an antigen-specific IgE assay, including those antigens showing some IgE reactivity in previous tests using a positive pool (data not shown). The antigens included in the IgE panel were: the Exported Protein 1 (EXP-1, Protein Potential), Merozoite Surface Protein 3 (MSP-3 3D7, ICGEB), Merozoite Surface Protein 2 3D7 strain type CH150 (MSP-2 3D7 CH150, Edinburgh University), CSP full length (Protein Potential), NANP repeat region (NANP, WRAIR) and C-term region (C-term, WRAIR).

2.3. Coupling of recombinant antigens to microspheres

MAGPLEX 6.5µm COOH-microspheres were purchased from Luminex Corporation (Austin, TX). The bead stock was gently resuspended on a rotary shaker for 30 min, followed by soft vortexing for 1 min and sonicated for 30 sec. The amount of beads to be coupled to each antigen was calculated assuming the use of 1,000 beads per region and per sample, and a maximum of 2.5 10^6 beads in 250µl reaction volume. The beads were washed twice with 250µl of distilled water in a concentration of 10,000 beads/µL by short vortex and sonication for 20 sec, and using a magnetic separator (Life Technologies, ref. 12321d). Next, the beads were resuspended in 80µl of activation buffer, 100mM monobasic sodium phosphate (Sigma, S2554), pH 6.2 by vortex and sonication for 20 sec. To activate the beads for cross-linking to proteins, 10µl of 50mg/ml Sulfo-N-hydroxysulfosuccinimide (Thermo Fisher Scientific, ref. 24525) and 10µl of 50mg/ml 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimidehydrochloride (Thermo Fisher Scientific, ref. 22981) were simultaneously added to the reaction tubes, mixed gently by vortexing and incubated for 20 min, at room temperature (RT), in a rotary shaker and protected from light. Microspheres were washed twice with 250µl 50mM morpholineethane sulfonic acid (MES) (Sigma ref. M1317) pH 5.0, or with phosphate buffered saline (Sigma ref. BE17-512F) pH 7.4 (Table 1), in a 10,000 beads/µL concentration by vortex and sonication for approximately 20 sec.

Table 1

Panel of *Plasmodium falciparum* and control antigens with their corresponding coupling concentration and the optimal coupling buffer.

Antigen	Coupling concentration	Buffer
AMA-1 3D7	20 µg/mL	MES
AMA-1 FVO	20 µg/mL	MES
MSP-1_{42} 3D7	20 µg/mL	MES
MSP-1_{42} FVO	20 µg/mL	MES
EBA-175	20 µg/mL	PBS
CelTOS	50 µg/mL	PBS
LSA-1	20 µg/mL	PBS
SSP2	10 µg/mL	PBS
DBL-α	30 µg/mL	PBS
CSP	10 µg/mL	MES
MSP-3 3D7	30 µg/mL	MES
MSP-2 3D7 CH150	30 µg/mL	MES
CSP C-term	30 µg/mL	MES
CSP NANP	30 µg/mL	MES
EXP-1	30 µg/mL	MES
BSA	PBS-BSA1%	PBS

Beads were first conjugated to the different antigens in a range of concentrations between 10-50 µg/mL to choose the optimal one for couplings. Then, the appropriate amount of each antigen per million beads (Table 1) was added to each reaction tube in 500µl MES or PBS (10,000 beads/uL), and they were left on a rotary shaker overnight at 4ºC, protected from light. Next day, beads were brought to RT in agitation for 20 min, and were blocked with 500µl PBS-BN (PBS with 1% BSA (Santa Cruz, SC-2323A) and 0.05% sodium azide (Sigma ref. S8032)) in agitation during 30 min at RT and protected from light. Beads were washed twice with PBS-BN by short vortex and sonication for 20 sec and using the magnetic separator. To determine the percentage recovery after the coupling procedure, coupled

beads were resuspended in 500µl PBS-BN and counted on a Guava PCA desktop cytometer (Guava Technologies, Automated cell counter, PC550IG-C4C / 0746-2747).

Antigen-coupled beads were validated in singleplex and multiplexed by measuring total IgG in serial dilutions of the positive control. Coupled beads were stored multiplexed at 1,000 beads/µl/region at 4ºC and protected from light.

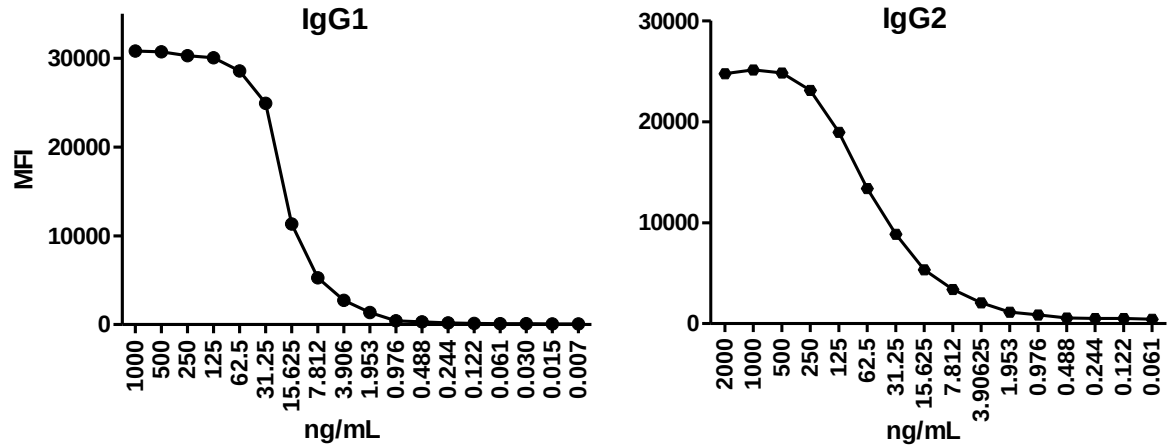
2.4. Coupling of anti-IgM, anti-IgG and anti-IgE antibodies to microspheres for the standard curves

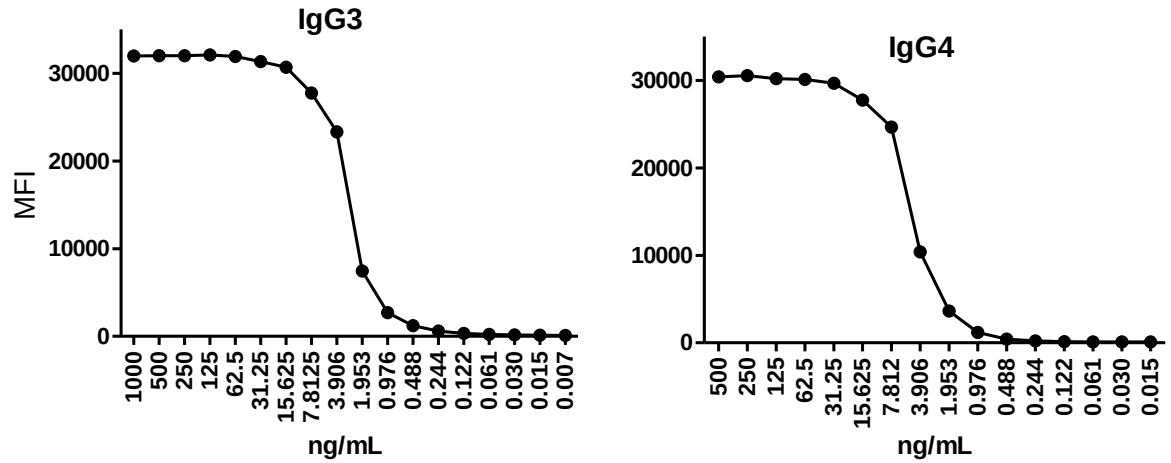
Anti-human IgM (KPL, ref.201-1003), anti-human Fab-IgG (Jackson Immunoresearch, ref. 109-006-097) and anti-human IgE (Abcam, ref. ab99834) antibodies were separately coupled to microspheres at 50 µg/mL following the coupling procedure above indicated. Antibody-coupled beads were tested in singleplex with serial dilutions of the corresponding human purified immunoglobulin: IgM (Sigma-Aldrich, ref. I8260), IgG1 (Abcam, ref. ab90283), IgG2 (Abcam, ref. ab90284), IgG3 (Abcam, ref. ab138703), IgG4 (Abcam, ref. ab90286) and IgE (Abcam, ref. ab65866). Antibody-coupled beads for the standard curves were stored at 2,000 beads/µl at 4ºC and protected from light. Anti-human IgE coupled beads were also used to measure total IgE in the test samples.

2.5. General assay procedures

Several biotinilated secondary antibodies were tested against the positive and negative controls to assess their ability to detect IgM, IgG1, IgG2, IgG3, IgG4, and IgE in plasma samples. In parallel, for each specific assay we evaluated the best combination of primary and secondary antibody pairs to create a standard curve of serial dilutions of the corresponding human purified isotype/subclass (Fig. 1). Standard curves of antibody concentrations versus MFIs were fitted using a 5-parameter (5PL) or a 4-parameter (4PL) logistic equation depending on the best yield (superior fit to antibody data). If 5-PL regression model did not converge, then a 4-PL method without asymmetry factor was fitted instead, following the formula MFI=Emax+((Emin-Emax)/((1+((Conc/EC50)^Hill))^Asym)), where EC_{50}

is the half maximal effective concentration, Emin is the minimum response, Emax is the maximum response, Asym is the asymmetry factor and Hill is the slope factor. Titration of antibodies and optimal sample dilutions were assessed through several tests.





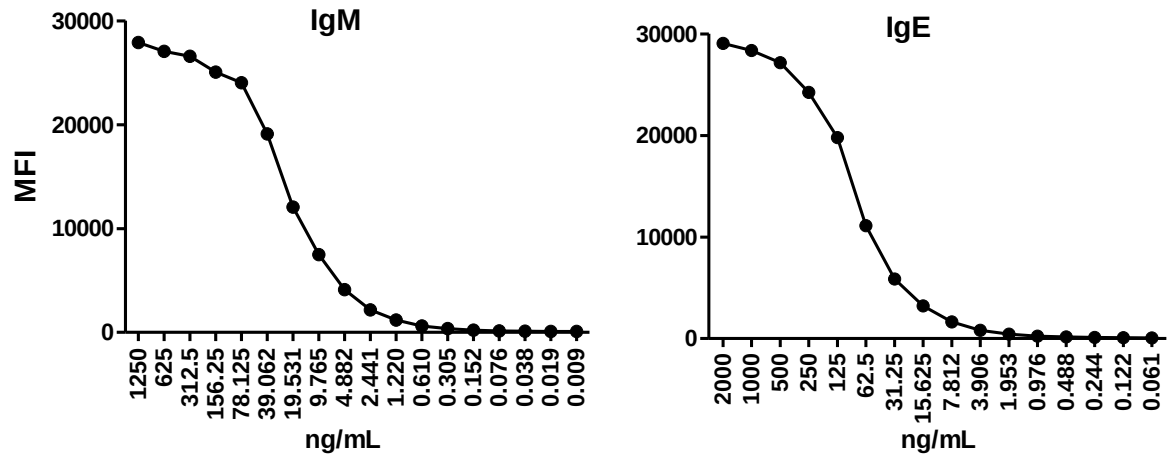


Fig. 1. Examples of IgG1, IgG2, IgG3, IgG4, IgM and IgE standard curves prepared with serial dilutions of the corresponding human purified isotype/subclass.

Regarding the general assay procedures, we followed the previously described total IgG protocol with some modifications[32, 47]. First, antigen-coupled microspheres were added to a 96-well µClear® flat bottom plate (Greiner Bio-One, ref. 655096) in multiplex (1,000 microspheres per analyte per well)[48] in a volume of 50µL of PBS-BN and incubated with 50µL of serial dilutions of the positive control (usually between the range 1/10-1/400,000) and the negative controls (at the first dilution of the positive control). A couple of wells per plate were designated to blanks where beads were incubated with PBS-BN to measure background signal. As standard curve, anti-IgM, anti-IgG or anti-IgE coupled microspheres were added in singleplex (2,000 microspheres per well) in a volume of 50µL of PBS-BN and incubated with 50µl of 2-fold serial dilutions of the corresponding purified human IgM, IgG1, IgG2, IgG3, IgG4 or IgE. Next, plates were incubated during 1h at 600 rpm in a microplate shaker (Corning, LSE Digital Microplate Shaker ref. CLS67814-1EA) at RT and protected from light. After the incubation, plates were washed manually three times with 200µl/well of wash buffer (PBS-Tween20 0.05%) on a magnetic washer (Millipore, ref. 40-285). A hundred microliters of biotinilated secondary antibody (anti-human IgM, IgG1, IgG2, IgG3, IgG4 or IgE) diluted in PBS-BN were added to all wells and incubated 45 min at 600rpm, RT and protected from light. The plate was washed as before and 100µl of streptavidin-R-phycoerythrin (Sigma, ref. 42250) diluted 1:1,000 in PBS-BN were added to all wells and incubated 30 min at 600rpm, RT and protected from light. Plates were washed three times as before, and beads resuspended in 100µl of PBS-BN and immediately read using the Luminex xMAP® 100/200 analyzer. At least 50 beads per analyte were acquired per sample. Crude median fluorescent intensity (MFI) and background fluorescence from blank wells were exported.

2.6. Use of a tertiary antibody in the antigen-specific IgG2 and IgE assays

The double antibody sandwiches tested for the antigen-specific IgG2 and IgE assays did not render enough signal for the measurement of these two antibodies. To increase the sensitivity of the assays we added a tertiary antibody. For these triple sandwiches, the secondary antibodies used were unconjugated mouse anti-human IgG2 and anti-human IgE diluted 1/500 in PBS-BN. The secondary antibody was incubated 60 min, followed by an incubation with anti-mouse IgG-Biotin diluted 1/1,000 in PBS-BN for 60 min, and a last incubation with streptavidin-R-phycoerythrin diluted 1:1,000 in PBS-BN 30 min. Incubations and washes in-between were as indicated in the previous section.

2.7. Assay reproducibility

For each isotype and IgG subclass, the coefficient of variation (CV%) was assessed for the duplicates and for the repeated measurements of the positive control in different plates. Means of CV% of duplicates per antigen and isotype-subclass were calculated.

3. Results

In the optimization of the IgGs and IgE assays, many different reagents and antibody sandwiches were tested, some of them discarded because there was no recognition of their expected target, or due to unspecific binding to other IgG subclasses or even to the antigen-coupled beads. Here we present the antibody combinations that better detected antigen-specific antibodies in human plasma or serum samples and that better worked for the development of the corresponding standard curves.

3.1. Standard curves

The primary human purified Ig used for the development of the standard curves are shown in Table 2, where serial dilutions are detailed. Figure 1 shows examples of the performance of each standard curve.

Table 2

Details of primary antibody dilutions used for the development of the standard curves.

Human purified Ig isotype/subclass	Fold dilution	Starting concentration (ng/ml)	Final concentration (ng/ml)	Serial dilutions
IgM (Sigma-Aldrich, I8260)	2	1250	0.009536743	18
IgG1 (Abcam, ab90283)	2	1000	0.00762939	18
IgG2 (Abcam, ab90284)	2	2000	0.06103516	16
IgG3 (Abcam, ab138703)	2	1000	0.00762939	18
IgG4 (Abcam, ab90286)	2	500	0.01525879	16
IgE (Abcam, ab65866)	2	2000	0.06103516	16

3.2. Optimal antibody sandwiches and dilutions for each Ig isotype/subclass assay

The primary antibodies used for the optimization of the assays were the same used for the standard curves (Table 2). For the selection of the secondary and tertiary antibodies, the main criterion used was the generation of good standard curves. Antibodies selected were titrated to find optimal assay dilutions (Table 3).

Table 3

Details of secondary-tertiary antibodies and Streptavidin-Phycoerythrin (SA-PE) reagents.

Ig	Secondary antibody	Tertiary antibody	SA-PE
IgG1	anti-IgG1-Biotin (Abcam, ab99775) 1:4000	-	Streptavidin-R-phycoerythrin (Sigma, 42250) 1:1000
IgG2	mouse anti-human IgG2 (Thermo Fisher, MA1-34755) 1:500	goat anti-mouse IgG-Biotin (Sigma, B7401-1ML) 1:1000	
IgG3	anti-IgG3-Biotin (Sigma, B3523) 1:1000	-	
IgG4	anti- IgG4-Biotin (Sigma, B3648) 1:8000	-	
IgM	anti-IgM-Biotin (Sigma, B1265) 1:1000	-	

Antigen-specific IgE	Mouse anti-human IgE (Abcam, ab99834) 1:250	goat anti-mouse IgG-Biotin (Sigma, B7401-1ML) 1:125
Total IgE	anti-IgE-Biotin (Thermo Fisher, A18803) 1:2000	-

The human purified IgM captured by anti-IgM-coupled beads was detected by anti-IgM-Biotin (Sigma, B1265) diluted 1:1000. The human purified IgG1, 3 and 4 subclasses separately captured by anti-IgG-coupled beads were detected by anti-human IgG1-Biotin (Abcam, ab99775) at dilution 1:4,000, anti-human IgG3-Biotin (Sigma, B3523) at dilution 1:1,000 and anti-human IgG4-Biotin (Sigma, B3648) at dilution 1:8,000, respectively. For the IgG2 assay, after testing many different reagents, suppliers and incubation times, we did not find any biotinilated secondary antibody that performed properly. We solved the problem by using a triple sandwich including a secondary plus a tertiary biotinilated antibody: an unconjugated mouse anti-human IgG2 (Thermo Fisher, MA1-34755) diluted 1:500 and a goat anti-mouse IgG-Biotin (Sigma, B7401-1ML) diluted 1:1,000. The same situation happened for the antigen-specific IgE assay, therefore we chose as secondary antibody an unconjugated mouse anti-human IgE (Abcam, ab99834) diluted 1:250 and a goat anti-mouse IgG-Biotin (Sigma, B7401-1ML) diluted 1:125 as tertiary antibody. Separately, for an assay measuring total IgE, the primary antibody was captured by anti-IgE coupled beads and detected by anti-human IgE-Biotin (Thermo Fisher, A18803) diluted 1:2000. For all of the assays, the streptavidin-PE incubation was optimal at a 1:1000 dilution. Once all assays were developed, we decided to also test a triple sandwich for IgG4 to make it more comparable to IgG2, the other minority non-cytophilic subclass. The IgG4 triple sandwich was successfully developed, and antibodies and dilutions finally selected were: a mouse anti-human IgG4 (Thermo Fisher, MA1-80332) diluted 1:8,000 and the goat anti-mouse IgG-Biotin (Sigma, B7401-1ML) diluted 1:1,000.

3.3. Optimal plasma dilutions for each isotype/subclass assay

To determine the optimal sample dilutions, plasmas from 30 Mozambican individuals were tested in serial dilutions chosen depending on the expected levels for each Ig against *P. falciparum* antigens. Samples were diluted from 1:200 to 1:204,800 for IgM (Fig. 2), 1:400 to 1:1,638,400 for IgG1, 1:200 to 1:437,400 for IgG3 (Fig. 3), 1:50 to 1:12,800 for IgG2 and IgG4 (Fig. 4), and 1:10 to 1:1,771,470 for antigen-specific IgE and 1:50 to 1:25,600 for the total IgE assays (Fig. 5). To test the triple sandwich IgG4 assay, serial dilutions (1:100 to 1:13,107,200) of a pool of plasmas from hyperimmune adults were tested (Fig. 4).

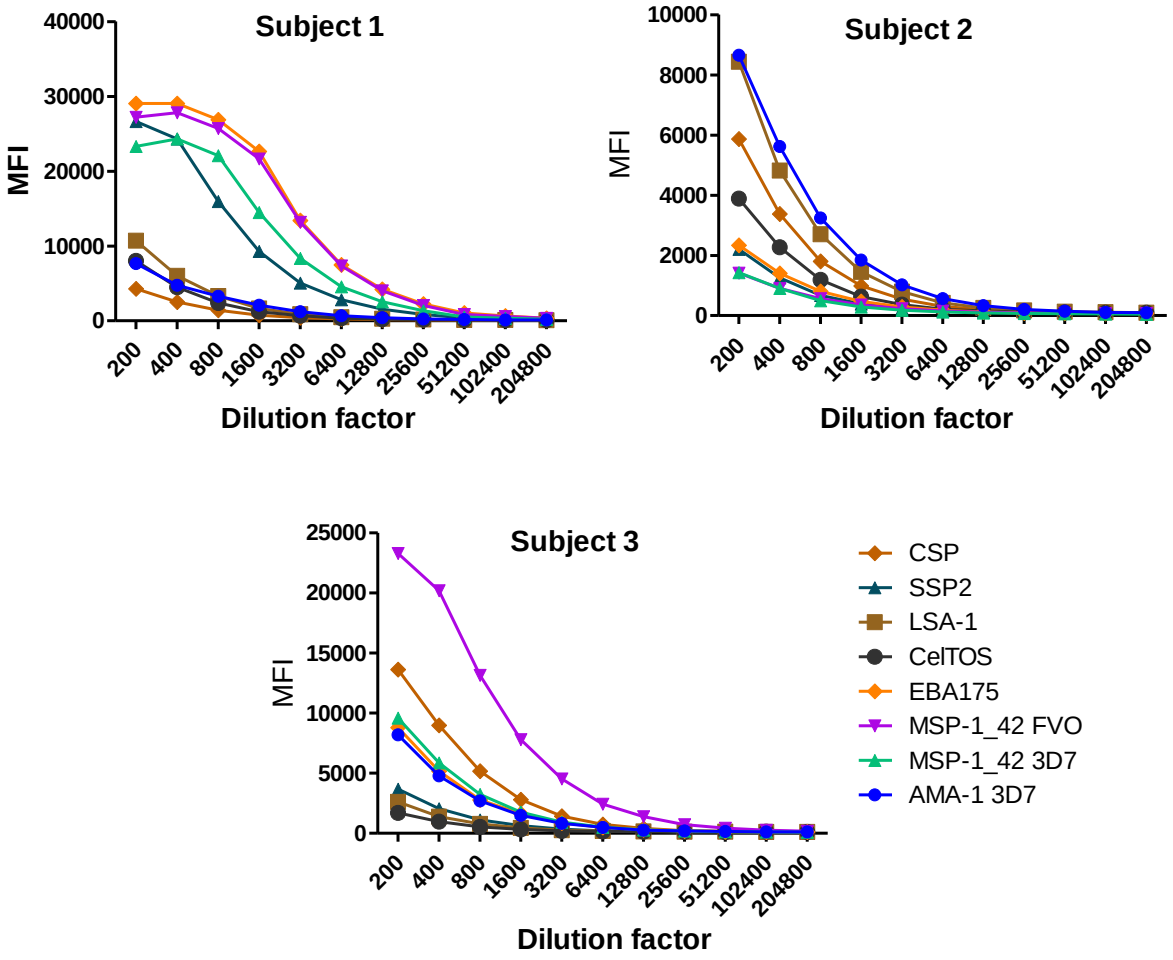
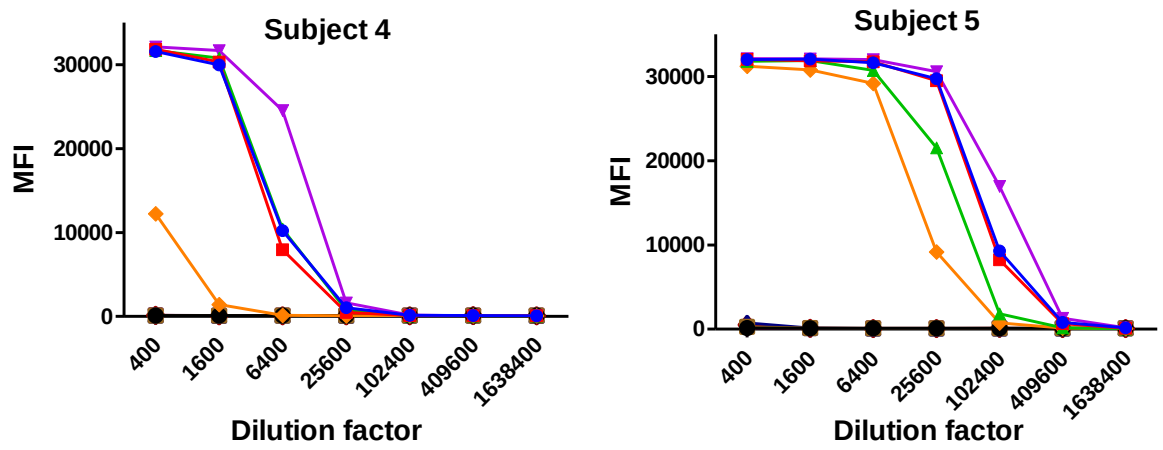


Fig. 2. Example of IgM levels measured in serial dilutions of plasma samples from 3 Mozambican adults against a panel of 8 *Plasmodium falciparum* antigens using anti-IgM-Biotin diluted 1:1,000 and streptavidin-PE at 1:1,000.

IgG1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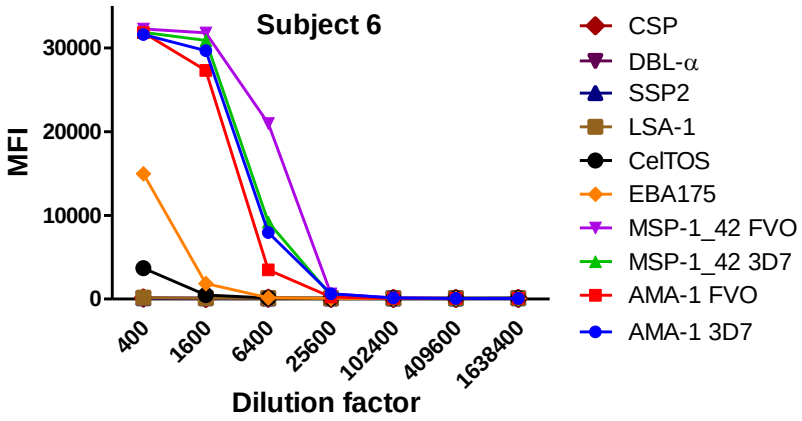
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IgG3

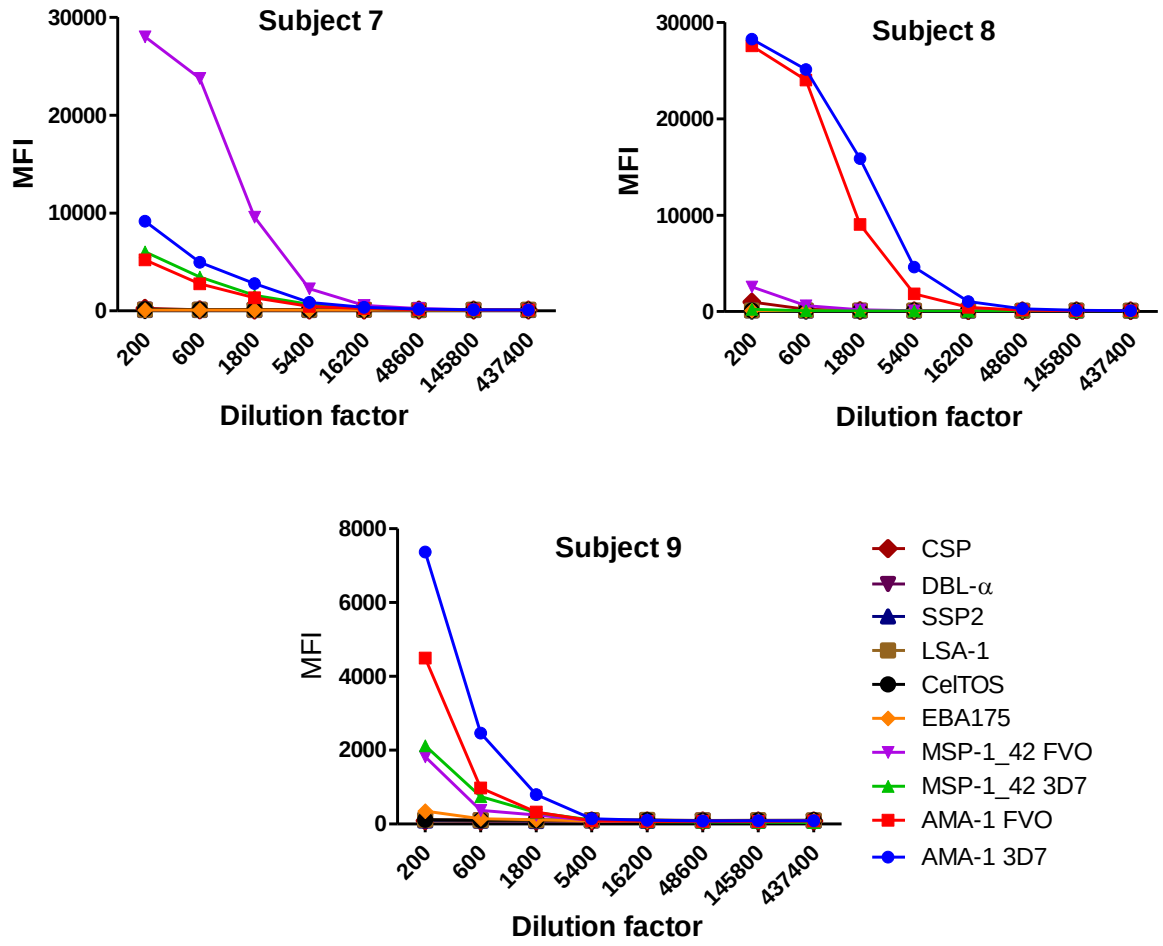
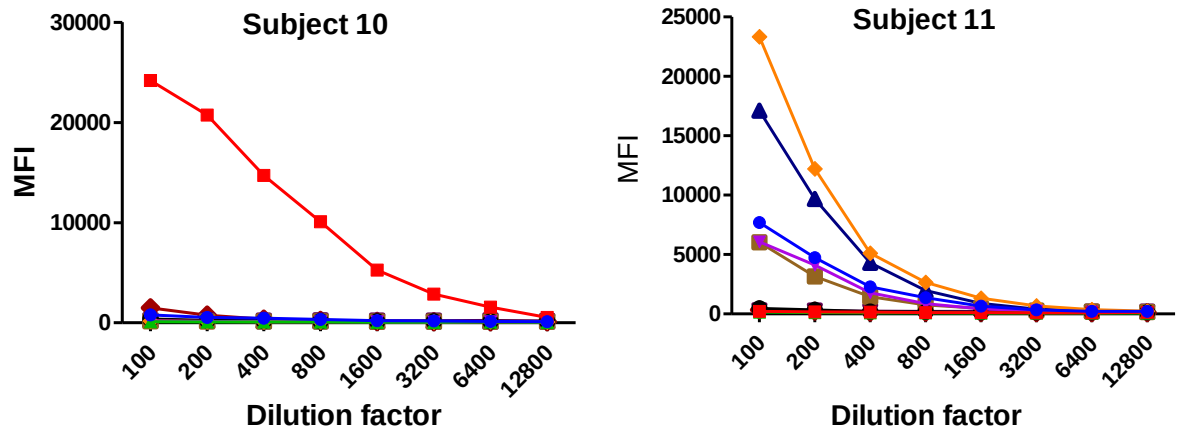
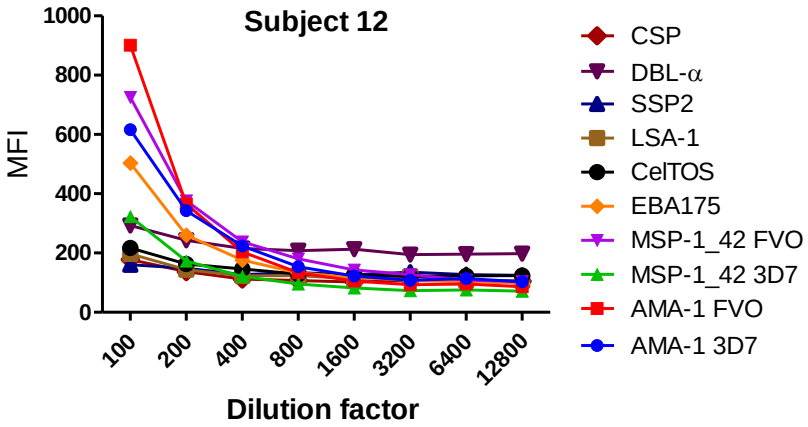


Fig. 3. Examples of IgG1 (subjects 4,5 and 6) and IgG3 (subjects 7, 8 and 9) levels measured in serial dilutions of plasma samples from 6 Mozambican adults against a panel of 10 *Plasmodium falciparum* antigens using anti-human IgG1-Biotin at 1:4,000 and anti-human IgG3-Biotin at 1:1,000, respectively, and streptavidin-PE at 1:1,000.

IgG2





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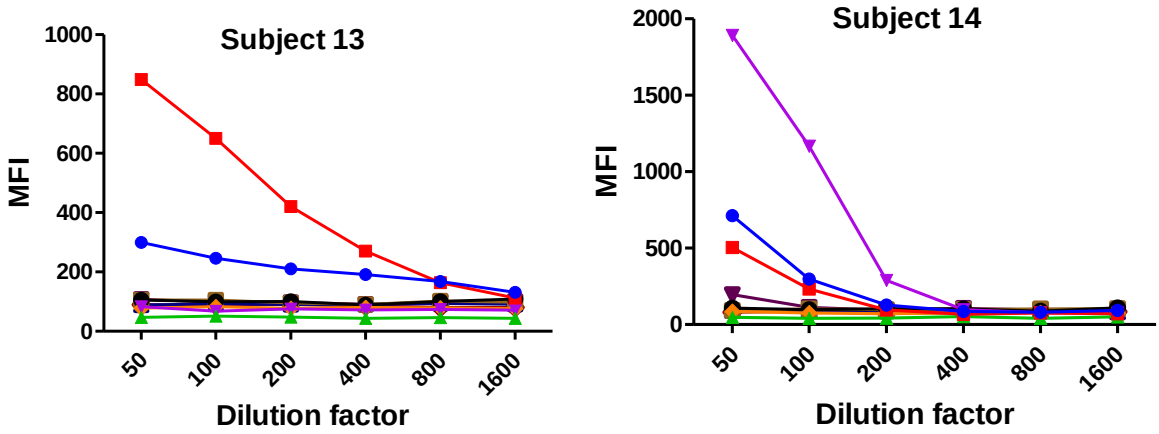
333 **IgG4 (double sandwich)**



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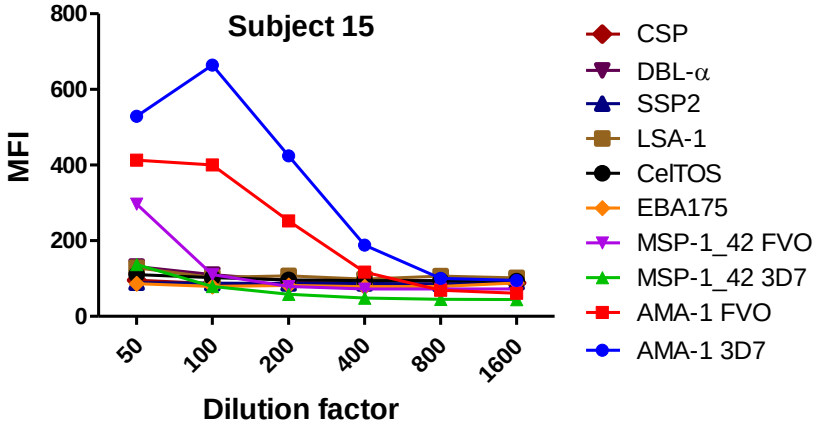

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336 **IgG4 (triple sandwich)**

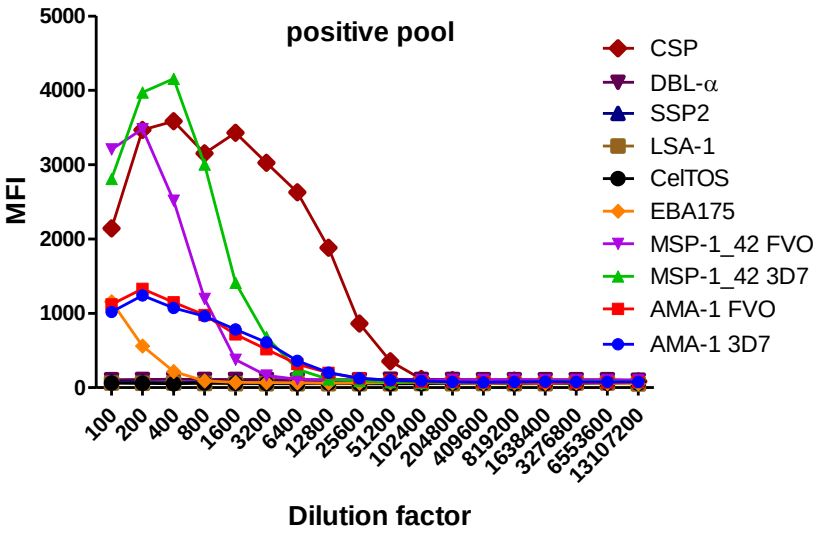
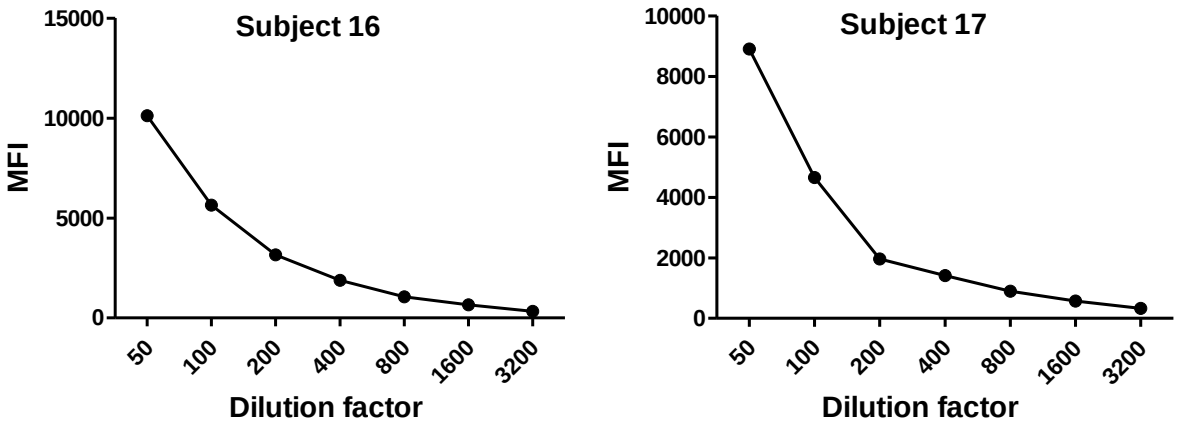
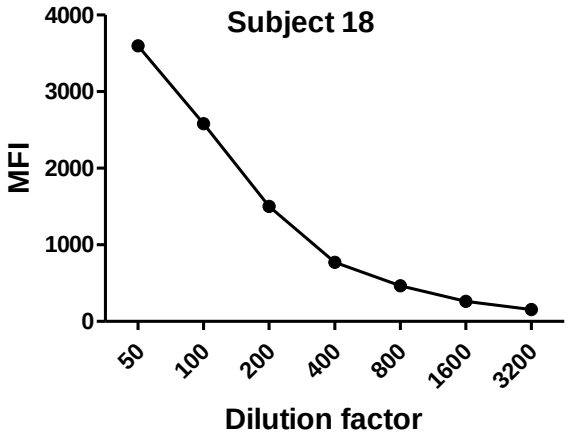


Fig. 4. Examples of IgG2 (subjects 10, 11 and 12) and IgG4 (subjects 13, 14 and 15) levels measured in serial dilutions of plasma samples from 6 Mozambican adults against a panel of 10 *Plasmodium falciparum* antigens using a triple (mouse anti-human IgG2 at 1:500 plus goat anti-mouse IgG-Biotin at 1:1,000) and double sandwich (anti-human IgG4-Biotin at 1:8,000), respectively, and incubated with streptavidin-PE at 1:1,000. Data obtained using a triple sandwich (mouse anti-human at 1:8,000 plus goat anti-mouse IgG-Biotin at 1:1,000) to measure IgG4 in a pool of plasmas from hyperimmune adults are also shown.

Total IgE





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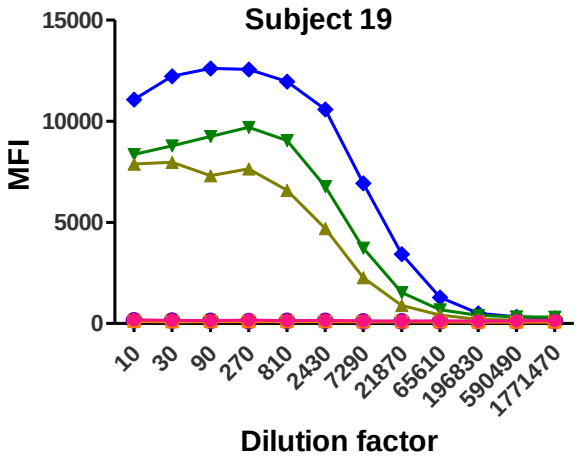
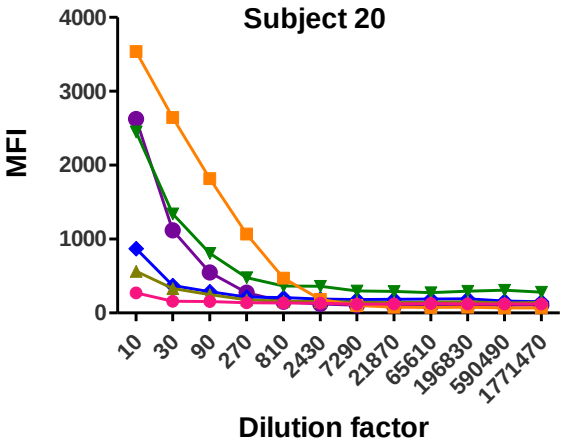
354 **Antigen-specific IgE**





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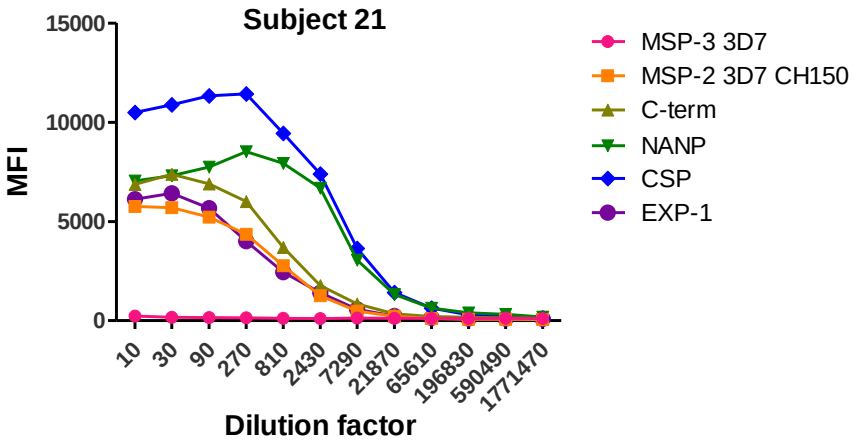
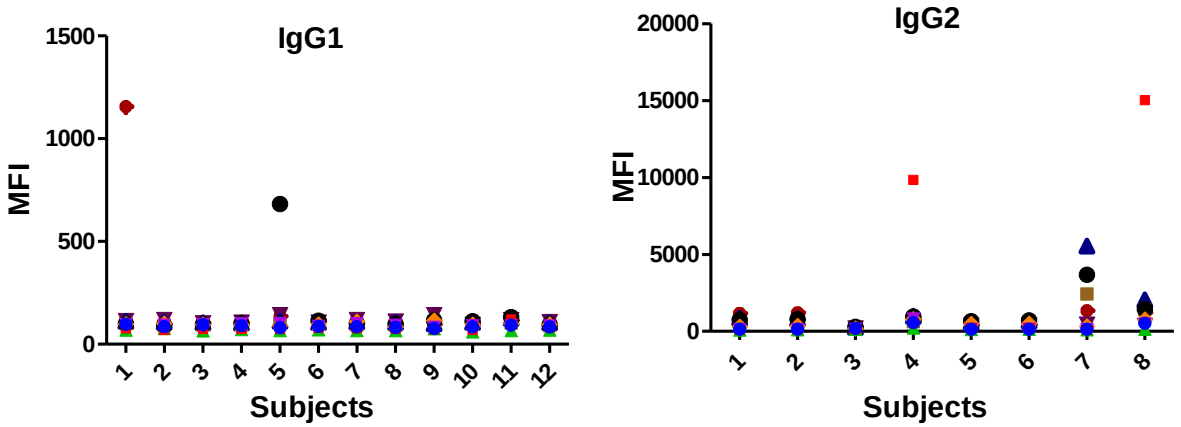


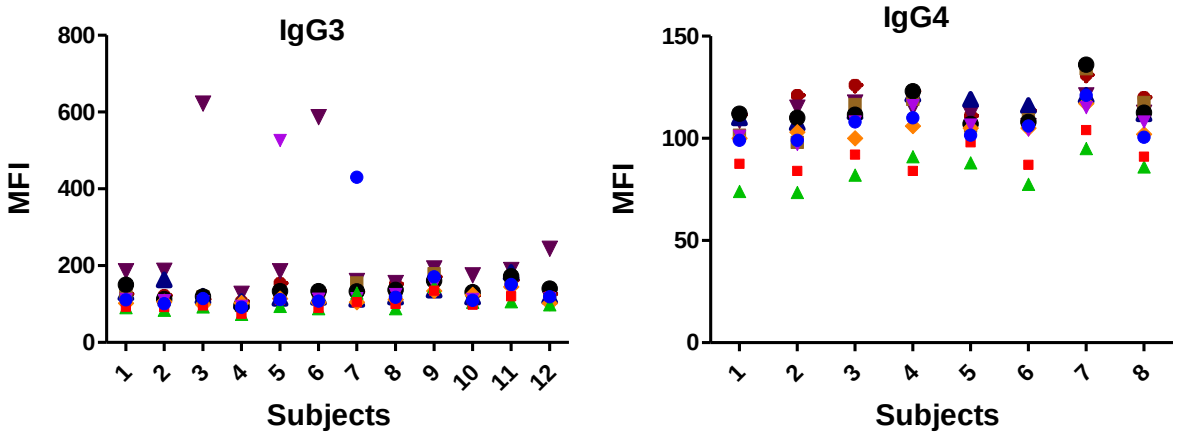
Fig. 5. Total (subjects 16, 17 and 18) and antigen-specific (subjects 19, 20 and 21) IgE levels measured in serial dilutions of plasma samples from 6 Mozambican children. Total IgE has been measured using an anti-human IgE-Biotin diluted 1:2,000. IgE antigen-specific levels have been measured against a panel of 6 *Plasmodium falciparum* antigens using an unconjugated mouse anti-human IgE diluted 1:250 and a goat anti-mouse IgG-Biotin diluted 1:125. Streptavidin-PE was incubated at a 1:1,000.

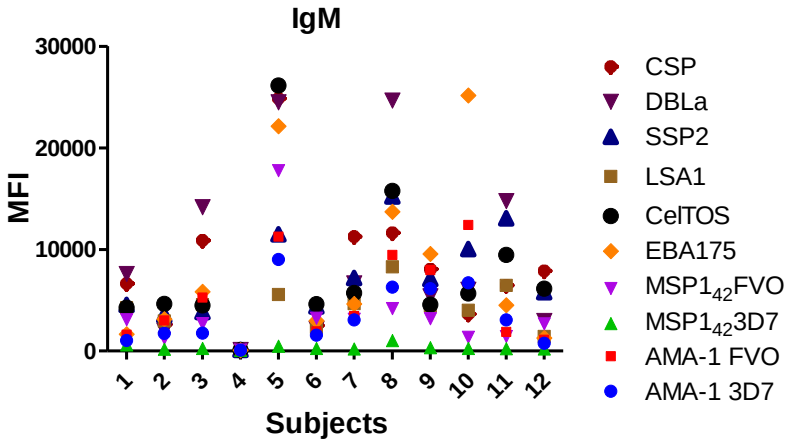
IgM, IgG1 and IgG3 individual responses to the different antigens were of very different magnitudes (Fig. 2 and 3). IgG2, IgG4 and IgE responses in almost all the tested individuals were overall weak (Fig. 4 and 5). Accordingly to these results, for IgM, IgG1 and IgG3 we concluded to work with at least two dilutions per sample to increase the chances of one falling in the linear part of the standard curve, assuring reliable measurements. On the other hand, for IgG2, IgG4 and total IgE, we concluded that only one plasma dilution was needed because of the low signals observed. For the antigen-specific IgE assay, we decided to use 4 serial dilutions, being an almost unexplored isotype in the malaria field. Therefore, we chose 1:200 and 1:20,000 sample dilutions for IgM assay; 1:400 and 1:12,000 for IgG1; 1:200 and 1:1,000 for IgG3; 1:50 for IgG2, IgG4 and total IgE; and 1:30, 1:270, 1:2,430 and 1:21,870 for specific IgE.

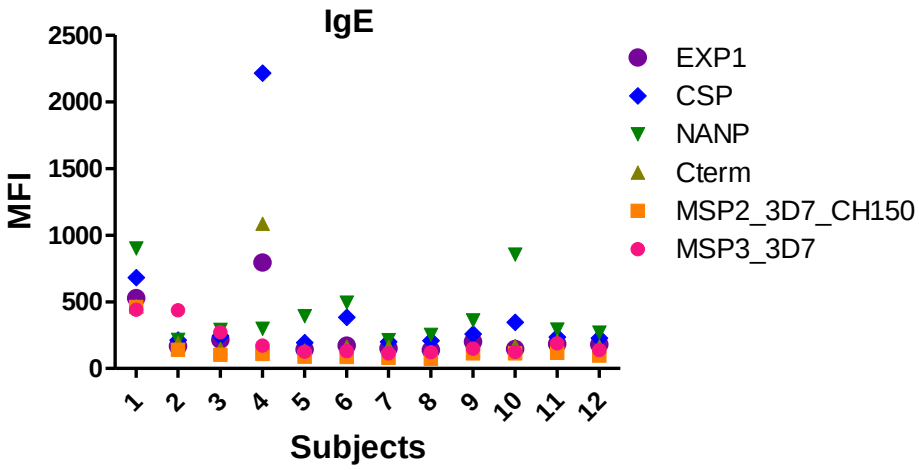
The positive control was assayed in the same dilutions as the samples. For the negative controls (European naïve individuals) the dilution chosen was the samples' most

concentrated for each isotype/subclass. Figure 6 shows examples of antibody levels in plasma samples from negative controls. Some malaria naïve individuals had a signal against some *P. falciparum* antigens, probably because of cross-reactivity of non-specific antibodies. For IgM, unspecific responses of negative controls were higher than for the other antibodies.









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388 **Fig. 6**. Example of levels of antigen-specific IgG1, IgG2, IgG3, IgG4, IgM and IgE measured
389 in plasma from European naïve individuals, at the dilutions 1:400, 1:50, 1:200, 1:50, 1:200
390 and 1:30, respectively.

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392 All 6 antigen-specific Ig assays showed very low background signals measured in blank wells
393 (Fig. 7), being the antigen-specific IgE assay the one with higher MFIs when no sample was
394 incubated.

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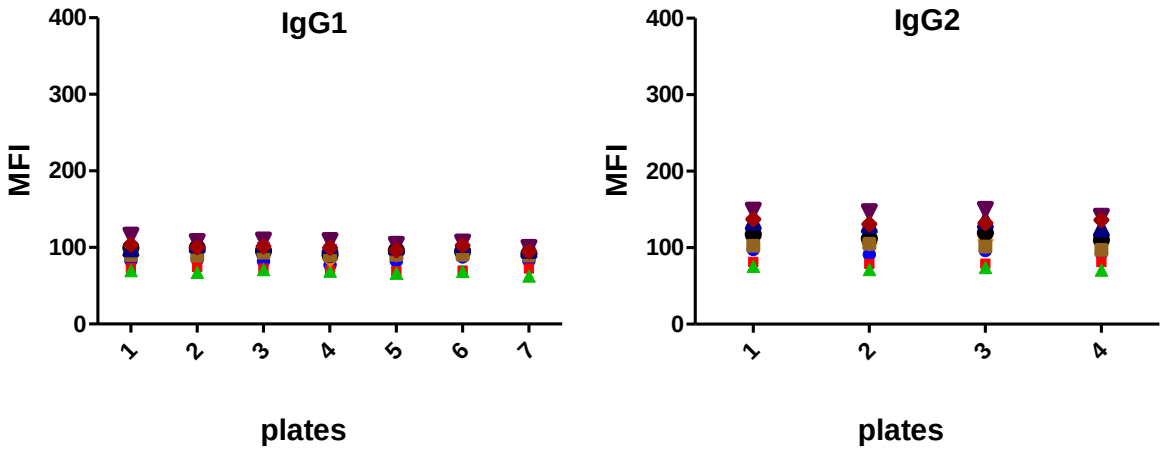

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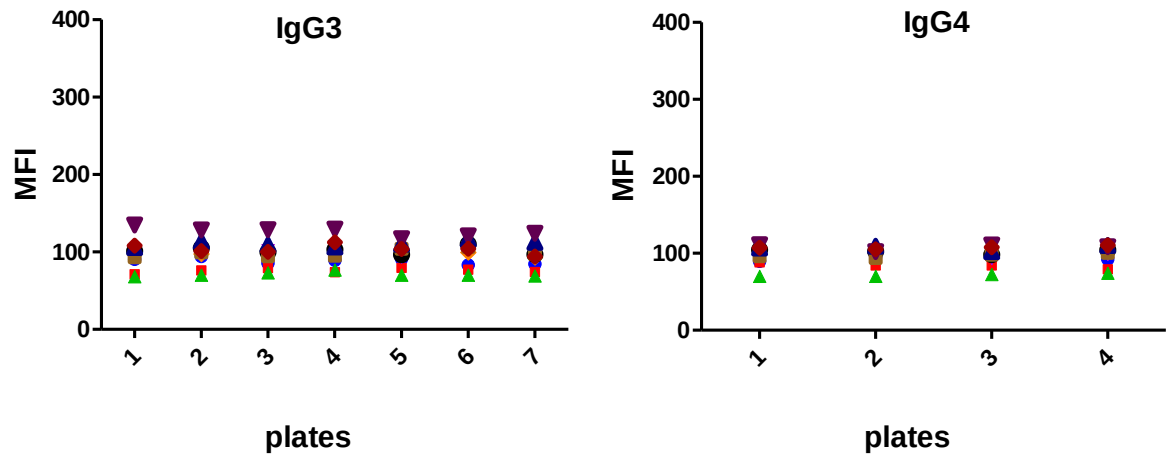

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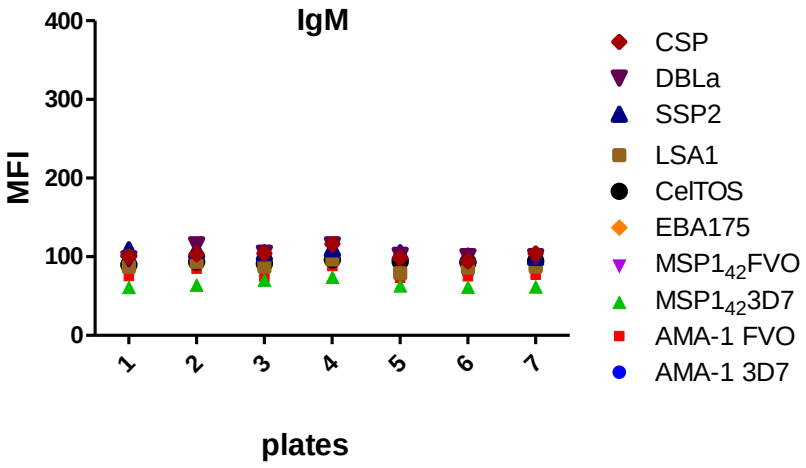

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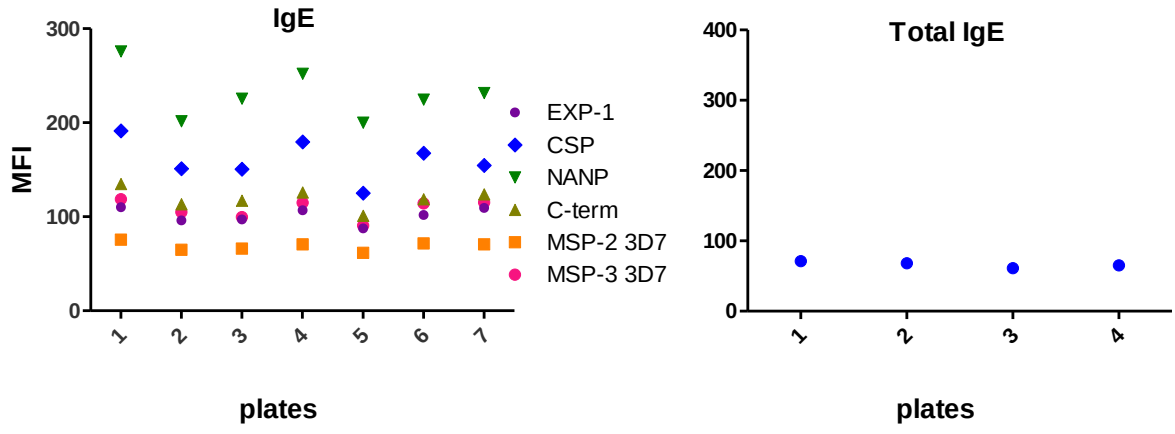

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400 **Fig. 7.** Levels of background signal in each antigen-specific isotype/subclass assay.

401

402 *3.4. Reproducibility intra- and inter-plate*

Data from the positive controls (two dilutions for IgM, IgG1 and IgG3, one dilution for IgG2 and IgG4, and 4 dilutions for IgE) were tested in duplicates during 2 consecutive days in 7 different plates to assess the reproducibility of the assay between duplicates and plates. CVs between duplicates were 3.32% (1.13-5.09) for IgM, 3.27% (0.32-6.08) for IgG1, 3.98% (1.6-7.61) for IgG2, 3.72% (0.41-6.65) for IgG3, 3.66% (1.03-5.73) for IgG4, 4.45% (3.14-5.44) for specific IgE and 2.86% (2.26-3.47) for total IgE. The overall CV was 3.47%, indicating that the measurements were consistent. The CVs between different plates are shown in Table 4 (IgG1, IgG2, IgG3, IgG4 and IgM) and Table 5 (IgE) with CVs ranging from 1.55% to 27.58%.

Table 4

Coefficients of variability of repeated measurements of *Plasmodium* antigen-specific IgG1, IgG2, IgG3, IgG4 and IgM assayed in the positive control.

Ig	Dilution	AMA-1 3D7	AMA-1 FVO	MSP-1_{42} 3D7	MSP-1_{42} FVO	EBA-175	CelTOS	LSA-1	SSP2	DBL-α	CSP
IgG1	1	1.75	1.55	5.16	2.3	20.02	6.58	11.76	6.34	10.12	12.64
	2	5.01	7.22	9.75	15.01	4.49	3.33	3.2	3.13	3.68	3
IgG2	1	8.93	7.33	9.65	13.33	4.9	8.24	12.6	8.56	9.67	9.31
IgG3	1	1.59	1.7	11.53	3.14	7.65	12.45	19.68	27.4	15.12	26.41
	2	14.54	11.03	20.33	18.68	21.09	5.42	7.64	19.55	4.69	18.75
IgG4	1	4.68	7.08	4.02	2.96	6.21	2.8	5.21	5.05	5.75	2.42
IgM	1	21.78	19.86	18.27	7.69	18.75	16.32	13.14	18.95	16.9	13.22
	2	20.62	22.63	19.44	27.58	24.71	19.5	16.42	21.23	24.36	21.57

Table 5

Coefficients of variation. **5A.** Repeated measurements of antigen-specific IgE assayed with the positive control pool. **5B**. Repeated measurements of total IgE.

A.

Ig	Dilution	MSP-3 3D7	MSP-2 3D7 CH150	C-term	NANP	CSP	EXP-1
Antigen-	1	16.4	13.51	14.43	16.15	17.38	16.27

specific IgE	2	11.06	5.99	9.79	8.52	7.35	10.45
	3	11.92	17.03	9.89	8.06	6.97	18.38
	4	9.55	13.71	17.15	14.16	13.28	18.47

B.

Ig	CV%
Total IgE	5.24

4. Discussion

The identification of the antibodies generated after infection or vaccination, their magnitude, and their antigen specificity, are essential to improve the development of more efficient vaccines against malaria. With this purpose, we have developed six qSAT protocols to measure antigen-specific IgM, IgG1, IgG2, IgG3, IgG4 and IgGE against *P. falciparum* antigens.

Many studies in the field of human humoral response to *P. falciparum* have been conducted using the ELISA[24, 33, 49, 50] or qSAT[48 5, 51]. However, to our knowledge, this is the first time that the qSAT has been adapted to measure different antigen-specific Ig isotypes and subclasses, specifically IgM, IgG1, IgG2, IgG3, IgG4 and IgE using a multiplex panel of *P. falciparum* pre-erythrocytic and blood stage antigens.

The most difficult part during the development of the assays was finding the right combination of antibody pairs able to detect both the human purified Ig used in the standard curves and the natural Ig present in plasma samples. There are many commercial sources with a large catalogue of biotinylated secondary antibodies available and it is difficult to know where to start. Even with reagents referenced in published studies, we did not always get acceptable results. For some antibody combinations, we did not get any signal. For others, we got signals in the standard curves but not in the samples, or vice versa; others showed

high background signals. Another challenge we faced was that some secondary antibodies had very variable bath-to-batch activity, forcing us to titrate each new lot and always test for background signal. In the case of IgG2 and IgE assays, where we finally decided to use of a triple sandwich, a double titration was required including secondary and tertiary antibodies. All these requirements made the optimization of these assays a labor-intense and long process.

The use of a triple sandwich for IgG2 and IgE was chosen to increase the sensibility of the assays because the double sandwich yielded very poor signals. In addition, despite having developed a successful IgG4 assay using a double sandwich, the use of a triple sandwich also increased the sensibility, thus being adopted for subsequent studies.

Regarding assay reproducibility, the CVs between plates never reached 30% for any isotype/subclass, however it was between 20-30% for some antigens. To decrease the variability and increase the accuracy of the assays, future optimization efforts will explore modifying samples incubation times and temperatures.

Concerning assay specificity, the anti-*P. falciparum*-Ig signal detected in negative controls was overall low except in few subjects for IgG2, and especially for IgM, reaching high MFIs in some individuals. IgM has as a natural quality to be highly polyreactive against foreign and self-antigens, and it is thought to aid in the neutralization of pathogens prior to the development of high affinity, antigen-specific antibodies; it may also facilitate the clearance of apoptotic cells and/or autoantigen-immunocomplexes[52]. The possibility that this polyreactivity could provide some protection against pathogens that have not yet ben “seen” by the immune system of the host has been proposed[53, 54]. Thus, even in the naïve population (as are our negative controls) unexpected elevated levels of IgM with these characteristics can be detected. A cross-reactivity of IgM with antigens from other pathogens to which the negative controls have been exposed is another plausible explanation. Nevertheless, other negative controls will be tested in future IgM assays to find out if this was a problem with these specific negative controls or if it is something generalized. In parallel, other reagents, such as the biotinylated detection antibody, and other assay conditions like the temperature

of samples incubation, will be assessed to try to reduce the background signal and improve the assay performance. Nevertheless, the rest of assay background signals were low, between 61 and 276 MFI overall. All secondary and tertiary antibodies were selected because they showed levels below 300 MFI. Some reagents were discarded for having higher than expected background. The IgE-specific assay was the one with higher values but still considered in a good range.

The inclusion of a standard curve[55] in the assay is important as a quality control tool and applicable to choose the adequate sample dilution closer to EC_{50}, to be used in data preprocessing. The standard curve may also be used for the normalization of the data to correct the variability between plates. This can be done by using a dilution point in the linear part of the curve to calculate a correction factor as the ratio between the median of this point from all plates divided by the same point in the specific plate. The normalization factor is then isotype-specific. In addition, the standard curve can also be used to calculate concentrations in arbitrary units, as was done for IgG and IgM assays in prior studies[32, 47].

Regarding the selection of the sample dilutions optimal for each isotype/subclass assay, the choice will always depend on several factors: i) the demographic and clinical characteristics of the study population, (i.e. age, level of malaria exposure, pregnancy, treatment), ii) the objective of the study (i.e. to explore natural or artificially-acquired immunity through vaccination), and iii) the immunogenicity of the antigens in the study panel. In the case of *P. falciparum* immunoassays, and to assure that at least one sample dilution will always fall on the linear (quantifiable) part of the standard curve, we recommend to test them in at least two dilutions for IgM, IgG1 and IgG3, while only one dilution may be required for IgG2 and IgG4. Regarding total and antigen-specific IgE assays, we suggest 1 dilution for total IgE and 4 dilutions for antigen-specific IgE, since little is known about this isotype in malaria immunity. Nevertheless, we reiterate that depending on the characteristics of the study population and the immunogenicity of the antigens included in the panel, several serial dilution(s) of a representative set of samples have to be previously tested to guarantee an optimal choice

and avoid out-of-range MFI values in the qSAT, or other difficulties like the prozone or hook effect[56].

Beyond malaria, a great advantage of the qSAT is the possibility to optimize the measurement of different antibody isotypes and subclasses against all kinds of antigens from all kind of pathogens or conditions. This is a very powerful tool to address co-infections, a common situation in malaria endemic countries but often ignored when immune responses are explored and addressed individually.

5. Conclusion

A better characterization of the human immune response against *P. falciparum* is key to understand the mechanisms underlying protection, which in turn will allow the design of more effective vaccines. The 6 assays developed in this study demonstrate that the qSAT is a powerful mid-high throughput approach to evaluate antigen-specific responses of different Ig isotype/subclasses against multiplexed *P. falciparum* antigens. These 6 assays will allow performing detailed immuno-profilings against antigens from *P. falciparum* and other pathogens to better address natural and vaccine-induced humoral immune responses.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Data obtained in this study and more details are available from the corresponding author on reasonable request.

Ethics approval

Approval for the protocols was obtained from the Hospital Clínic of Barcelona Ethics Review Committee and the National Mozambican Ethics Review Committee.

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