

1 **Quantification of malaria antigens PfHRP2 and pLDH by quantitative suspension**
2 **array technology in whole blood, dried blood spot and plasma**

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33 dehydrogenase, Luminex, quantitative suspension array technology

34 **ABSTRACT**

35 **Background**

36 Malaria diagnostics by rapid diagnostic tests (RDTs) relies primarily on the qualitative
37 detection of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and *Plasmodium*
38 *sp* lactate dehydrogenase (pLDH). As novel RDTs with increased sensitivity are being
39 developed and implemented as point of care diagnostics, highly sensitive laboratory
40 based assays are needed for evaluating RDTs performance. Here, a quantitative
41 suspension array technology (qSAT) was developed, validated and applied for the
42 simultaneous detection of PfHRP2 and pLDH in a variety of clinical samples (whole
43 blood, plasma and dried blood spots) from different endemic countries.

44 **Results**

45 The qSAT was specific for the target antigens, with analytical ranges of 6.8 to 762.8
46 pg/ml for PfHRP2 and 78.1 to 17076.6 pg/ml for *P. falciparum* (Pf-LDH). The assay
47 detected *P. vivax* LDH (Pv-LDH) at a lower sensitivity than Pf-LDH (analytical range
48 of 1093.20 to 187288.5 pg/ml). Both PfHRP2 and pLDH levels determined using the
49 qSAT showed to positively correlate with parasite densities determined by quantitative
50 PCR (Spearman $r=0.59$ and 0.75 , respectively) as well as microscopy (Spearman $r=0.40$
51 and 0.75 , respectively), suggesting the assay to be a good predictor of parasite density.

52 **Conclusion**

53 This immunoassay can be used as a reference test for the detection and quantification of
54 PfHRP2 and pLDH, and could serve for external validation of RDTs performance, to
55 determine antigen persistence after parasite clearance, as well as a complementary tool
56 to assess malaria burden in endemic settings.

57

58 INTRODUCTION

59 The availability of field-deployable malaria rapid diagnostic tests (RDTs) in recent
60 years has markedly facilitated access to malaria diagnostics. Since the World Health
61 Organization (WHO) recommendations in 2010 to test all suspected malaria cases (1),
62 RDTs have gained a crucial role in the management of malaria clinical cases, as well as
63 for malaria surveillance. Malaria RDTs have supplanted conventional light microscopy
64 in many endemic areas as the standard of practice, accounting in 2017 for 75% of all
65 diagnostic tests done in sub-Saharan Africa, where most RDTs are distributed (66%) (2).
66 The vast majority of RDTs used worldwide are based on the detection of parasite
67 bioproduct histidine-rich protein 2 (PfHRP2), expressed only in *Plasmodium*
68 *falciparum*, and the parasite metabolic enzyme lactate dehydrogenase (pLDH), present
69 in all human-infecting *Plasmodium* species.

70 PfHRP2 is a water-soluble glycoprotein produced by the parasite throughout its asexual
71 lifecycle and early sexual stages; it is expressed on the surface of infected erythrocytes
72 and released into the peripheral blood circulation during schizogony (3,4). Given the
73 ability of mature *P. falciparum* parasites to sequester in vascular beds during the last
74 half of their asexual life-cycle, where they are not accessible for microscopic diagnosis,
75 it has been proposed that the quantitative detection of PfHRP2 can provide a more
76 accurate measurement of parasite biomass and potentially assist in determining the
77 prognosis of severe malaria (5–7). During pregnancy, *P. falciparum* infections can
78 remain undetectable in peripheral blood as the parasites sequester in the intervillous
79 spaces of the placenta by specific adhesion to chondroitin sulphate A (8,9). In such
80 scenario, PfHRP2-detecting RDTs have showed to have higher sensitivity on peripheral
81 blood compared to conventional light microscopy (10), although still lower than PCR
82 (11).

83

84 RDTs detecting PfHRP2 only are the most widely used products (12), accounting for
85 66% of the 276 million RDTs sold worldwide in 2017 (2). Nonetheless, PfHRP2-
86 detecting RDTs have been suggested to have limited clinical specificity for diagnosis of
87 current malaria infection in areas of high transmission (13) and following treatment
88 (14,15) due to the persistence of the protein in the blood circulation after parasite
89 clearance. The time span of a positive test result following parasite clearance is mainly
90 dependent on the duration and density of parasitaemia prior to treatment, with values
91 ranging from 26 days in Ugandan children with parasitaemia less than 1,000 parasites
92 per microliter (p/μl) up to 37 days for parasite density >50,000 p/μl (16).

93 The parasite LDH is a metabolic enzyme required for survival and is produced by all
94 five *Plasmodium* species infective to humans (17,18). In contrast to PfHRP2, pLDH
95 does not persist in blood after clearance of malaria infections and is therefore a better
96 marker of acute and current infection (19). Upon treatment, pLDH clearance in blood
97 has been shown to closely track with that of parasites, suggesting pLDH to be a suitable
98 predictor for treatment failure (20). However, sensitivity of RDTs based on this antigen
99 is generally lower than that of PfHRP2-based RDTs (21).

100 Currently, enzyme-linked immunoabsorbent assay (ELISA) is the standard of practice
101 immunoassay for the detection and quantification of PfHRP2 and pLDH, and is used as
102 an external validation tool for RDTs performance. ELISAs are however costly, time and
103 sample consuming, and generally only allow for the detection of one analyte at the time.
104 The recent release of a highly-sensitive RDT for PfHRP2 (AlereTM Malaria Ag P.f),
105 with two to ten-fold higher sensitivity than other currently available RDTs (22,23), as
106 well as the work in progress to develop new generation pLDH-based RDTs, underpin
107 the need for new highly-sensitive laboratory based reference immunoassays than can

108 provide lower limit of detection than classical ELISAs (24–28). Highly sensitive
109 quantitative assays should not only be a more suitable tool for validation of new-
110 generation RDTs, but could also be used to better understand antigen kinetics,
111 particularly that of PfHRP2, and to support malaria surveillance. In this work, we
112 present a high-throughput quantitative suspension array approach based on the Luminex
113 technology that allows for the simultaneous and highly sensitive detection and
114 quantification of PfHRP2 and pLDH antigens in different biological samples (whole
115 blood, plasma, and dried blood spots). This assay provides an additional tool to
116 externally evaluate the performance of new generation antigen detecting malaria RDTs,
117 and can be used for research purposes to address biological questions such as PfHRP2
118 persistence and the relationship between antigen levels and disease severity.

119

120 **MATERIALS AND METHODS**

121 **Development and optimization of the bead suspension array**

122 **Biotinylation of detection mAbs.** Detection monoclonal mouse IgG α -PfHRP2
123 (MBS834434, MyBioSource, San Diego, CA) and monoclonal mouse IgG α -PAN-
124 pLDH (PA-2, AccessBio, Somerset, NJ) were biotinylated using the EZ-Link Sulfo-
125 NHS-Biotin Kit (21435, Thermo Fisher Scientific, Waltham, MA) according to the
126 manufacturer's instructions with minor modifications (See Additional file 1: Text1).

127 **Coupling of mAbs to magnetic beads.** Coupling of magnetic microspheres was
128 performed similarly as described elsewhere (29). Briefly, two MagPlex® microspheres
129 (Luminex Corp., Austin, Texas) with different spectral signatures selected for the
130 detection of PfHRP2 and PAN-pLDH were washed with distilled water and activated

131 with Sulfo-NHS (N-hydroxysulfosuccinimide) and EDC (1-ethyl-3-[3-
132 dimethylaminopropyl] carbodiimide hydrochloride) (Pierce, Thermo Fisher Scientific
133 Inc., Rockford, IL), both at 50 mg/mL, in activation buffer (100 mM Monobasic
134 Sodium Phosphate, pH = 6.2). Microspheres were washed with 50 mM MES potassium
135 salt (4-morpholineethane sulfonic acid, Sigma Aldrich, St. Louis, MO) pH 5.0 to 10000
136 beads/ μ l, and covalently coupled with capture antibodies against PfHRP2 (MBS832975,
137 MyBiSource, San Diego, CL) and PAN-pLDH (PA-12, AccessBio, Somerset, NJ), both
138 at a concentration of 25 μ g/ml. Beads were incubated on a rotatory shaker overnight at 4
139 °C and protected from light. Microspheres were blocked with PBS-BN (PBS with 1%
140 BSA and 0.05% sodium azide (Sigma, Tres Cantos, Spain), and re-suspended in PBS-
141 BN (from now on named assay buffer) to be quantified on a Guava PCA desktop
142 cytometer (Guava, Hayward, CA) to determine the percentage recovery after the
143 coupling procedure. Coupling validation was performed by incubating 50 μ l of each
144 bead suspension (2000 beads/well) with 50 μ l α -mouse IgG-Biotin (B7401-1ML, goat
145 anti-Mouse IgG-Biotin, Sigma Aldrich, St. Louis, MO) at 1:1000 dilution in a 96-well
146 flat bottom plate for 2 hours in gentle agitation. The plate was washed by pelleting
147 microspheres using a magnetic separator (40-285, EMD Millipore, Burlington, MA) and
148 re-suspended with wash buffer (0.05% Tween 20/PBS). Beads were incubated with 100
149 μ l of streptavidin-phycoerythrin (42250-1ML, Sigma Aldrich, St. Louis, MO) diluted
150 1:1000 in assay buffer for 30 minutes in gentle agitation in the dark. Finally, the beads
151 were washed and re-suspended in assay buffer, and the plate was read using the
152 Luminex xMAP® 100/200 analyser (Luminex Corp., Austin, TX). A reading higher
153 than 25000 median fluorescence intensity (MFI) implied a successful coupling reaction.
154 Coupled beads were stored multiplexed at a concentration of 1000 beads/ μ l/region at 4
155 °C and protected from light.

156 To optimize the coupling concentration of detection antibodies, a concentration range
157 from 10 to 100 µg/ml of α-PfHRP2 and α-PAN-pLDH monoclonal antibodies (mAbs)
158 was conjugated to magnetic beads, and assayed against serially diluted recombinant
159 PfHRP2 and pLDH and a selection of plasma samples from *P. falciparum* positive
160 individuals. The mAb concentration that provided the highest the MFI values was
161 selected as the optimal concentration.

162 **PfHRP2 and pLDH reference materials.** Recombinant PfHRP2 protein type A from
163 FCQ79 *P. falciparum* strain expressed in *Escherichia coli* (890015, Microcoat GmbH,
164 Germany) was selected as PfHRP2 reference material. Antigen concentration after
165 reconstitution was determined by ELISA (Malaria Ag CELISA, CeLLabs). Purified
166 recombinant *P. falciparum* and *P. vivax* pLDH proteins expressed in insect cells (3001,
167 ReliaTech GmbH, Germany) were used as reference material. The pLDH concentrations
168 were measured in a previous study using a commercially available ELISA (QUALISA
169 Malaria kit, Qualpro Diagnostics, India) (21). Reference materials were used to prepare
170 the standard curves for the bead suspension array, starting at concentrations of 50 ng/ml
171 for PfHRP2 type A and at 1000 ng/ml for *P. falciparum* and *P. vivax* pLDH. The WHO
172 International Standard for *Plasmodium falciparum* antigens was provided by the
173 National Institute for Biological Standards and Control (Ridge, UK) (NIBSC code:
174 16/376, (30)). The WHO International Standard for *P. falciparum* antigens was
175 quantified, and the obtained antigen concentrations in pg/ml were used to calculate the
176 number of antigen picograms corresponding to 1 International Unit (IU).

177 **Optimization of assay standard curves.** Standard curves were prepared for the
178 detection of PfHRP2 and pLDH. The conjugated beads were incubated with serial
179 dilutions of recombinant PfHRP2 types A, B and C and recombinant *Pf* and *Pv* pLDH
180 in assay buffer to produce standard curves ranging from 50,000 to 0.024 pg/ml for

181 PfHRP2, and from 1,000,000 to 0.48 for both *P. falciparum* pLDH and *Pv* pLDH
182 (Figure 1B) (for a more detailed assay procedure, see Additional file 1: Text2).

183 **Assay parameters**

184 **Limit of detection, limits of quantification and range.** A calibration curve prepared
185 with serially diluted reference PfHRP2 and *P. falciparum* pLDH was assayed in 66 runs
186 on the Luminex xMAP® 100/200 analyser, along with 2 blank samples (consisting of
187 assay buffer alone) per run. For *Pv* pLDH, serial dilutions of reference antigen were
188 assayed in 6 independent runs. The lower limits of detection (LLOD), defined as lowest
189 amount of analyte which can be detected, and of quantification (LLOQ), defined as the
190 lowest concentration of an analyte in a sample that can be quantified, were determined
191 by measuring the MFI of 132 wells containing blank samples. The upper limit of
192 quantification (ULOQ), corresponding to the highest concentration that can be
193 quantitatively determined, was defined as the maximum value of the fitted mean
194 standard curve minus its 10% to avoid quantifying samples falling close to the
195 saturation plateau. The analytical range was set within the lower and the upper limits of
196 quantification.

197 To quantify the LLOD and the LLOQ, 3 and 6 standard deviations (SD) were added to
198 the mean MFI of blanks (n = 132), respectively. Each calibration or standard curve was
199 fitted using a five parameters logistic (5PL) regression, and the mean curve was
200 calculated. To present the LLOD and the LLOQ as concentration values, the calculated
201 MFI values were interpolated to the mean calibration curve.

202 **Dilution linearity and accuracy.** Dilution linearity and accuracy were evaluated on the
203 same serial dilutions of recombinant PfHRP2 type A and *P. falciparum* pLDH read over
204 66 independent runs. Dilution linearity was calculated as the mean percent change in

205 dilution-corrected concentration from one dilution to the previous one within the assay
206 range. Dilution linearity was considered acceptable if the percent change in
207 concentration did not exceed the recovery range of 80-120%. Accuracy was determined
208 as the mean percent deviation (% DEV) from the expected concentration, calculated by
209 dividing the difference between the experimental value and the expected value and then
210 multiplying by 100. Acceptable accuracy was defined as the %DEV not surpassing by
211 20% the expected concentration (by 25% for samples with concentrations falling at the
212 LLOQ and ULOQ).

213 **Precision.** Intra-assay and inter-assay precision were evaluated by assaying cultured *P.*
214 *falciparum* W2 strain spiked in assay buffer at five dilutions spanning a wide range of
215 antigen concentration in triplicate over four runs. Intra-assay precision over the four
216 runs was defined as the average coefficient of variation (% CV) of individual samples.
217 The % CV for each sample was calculated by determining the standard deviation (SD)
218 of the three replicate results, dividing it by the mean of the triplicate results, and
219 multiplying by 100. Inter-assay precision was defined as the overall % CV, calculated
220 by dividing the SD of plate means by the mean of plate means and then multiplying by
221 100. Calculations were performed on non-transformed MFI values. Precision was
222 considered acceptable when % CV did not exceed 10% for intra-assay variation and
223 20% for inter-assay variation (30).

224 **Selectivity.** To investigate the selectivity of the assay for the target antigens, 75 plasma
225 samples from 25 Spanish pregnant women never exposed to malaria were assayed to
226 demonstrate that the bead suspension array does not detect plasma components other
227 than the target antigens (PfHRP2 and pLDH).

228 **Study samples**

229 To test against samples collected in endemic areas, different sample sets were assayed
230 (characteristics of clinical samples used are summarized in Table 1).

231 ***Plasmodium falciparum* culture samples and *P. vivax* clinical samples.** W2, Benin I,
232 Borneo and Santa Lucia *P. falciparum* strains were cultured under standard hypoxic
233 conditions. Culture in exponential growth phase was harvested, infected red blood cells
234 were spun down, aliquoted, and frozen at -80 °C as previously described (21).
235 *Plasmodium vivax* isolates were collected from symptomatic adult volunteers with a *P.*
236 *vivax* mono-species infection as confirmed by microscopy during a specimen collection
237 campaign organized in April 2016 in the area of Iquitos (Peru).

238 **Plasma and serum samples.** PfHRP2 and pLDH were measured in 765 plasma
239 samples collected at 3 time points during pregnancy from 255 pregnant women residing
240 in Manhica (Southern Mozambique) who participated in a clinical trial of intermittent
241 preventive treatment during pregnancy (IPTp) from 2010 to 2012 (31,32), and in 103
242 serum samples from 77 pregnant women in the Urabá-Antioquia region (Colombia)
243 collected between 2005 and 2007 (33). Additionally, 75 plasma samples collected at 3
244 time points from 25 pregnant women never exposed to malaria, who attended the
245 Hospital Clínic of Barcelona during pregnancy and delivery in 2010, were included in
246 the assay as negative controls. Plasma and serum samples were stored at -80 °C.
247 Infection status and parasite densities were previously determined by qPCR on DBS for
248 samples from Mozambique (34), and by light microscopy (LM) in Colombian samples.

249 **Whole blood samples.** EDTA-anticoagulated whole blood samples were collected from
250 consenting asymptomatic adults with no recent clinical episode of malaria (past four
251 weeks) during cross-sectional surveys in Peru (35), and Senegal. Samples were assessed
252 and categorized as *P. falciparum* mono-species infection or *Plasmodium* negative
253 samples using nested PCR, and parasitaemia was quantified using quantitative PCR as

254 described previously at the Hospital for Tropical Diseases (UK) (36). The *pfhrp2* gene
255 status of *P. falciparum* PCR positive samples was investigated by PCR as previously
256 described (37). Whole blood samples from asymptomatic adults were used to prepare
257 dried blood spots (DBS) (See Additional file 1: Text4). EDTA-anticoagulated whole
258 blood samples were collected between March and October 2017 in Peru Amazon region
259 and Nigeria Lagos state from consenting symptomatic (with fever within the last 3 days)
260 and asymptomatic (no fever history in past 3 days) patients enrolled during a clinical
261 trial of a new multiplex fever diagnostic test. Antigens were quantified in those samples
262 that were positive for *P. falciparum* by PCR (n= 323 in Peru and 629 in Nigeria).
263 Individuals participating in this clinical trial had been previously tested by on-site
264 microscopy (final result based on reading from 2 independent microscopists), and by
265 SD BIOLINE Malaria Ag P.f (HRP2/pLDH) (05FK90, Abbott, Chicago, IL) in Nigeria
266 and by CareStart™ Malaria Pf/PAN (HRP2/pLDH) (RMRM-02571) and Carestart
267 Pf/PAN (pLDH) Ag (RMLM-02571) (AccessBio, Somerset, NJ) RDTs in Peru.

268 **Statistical analysis**

269 The relationship between the MFIs in singleplex and multiplex assays and the
270 correlation between parasite densities and antigen levels were assessed by the non-
271 parametric Spearman's rank correlation method. Statistical analyses were performed
272 with GraphPad Prism (version 6, Graphpad, Inc). The 95% confidence intervals (CI
273 95%) for sensitivity and specificity were calculated by Wilson score method in
274 Microsoft Excel (2013).

275

276 **RESULTS**

277 **Development of the bead suspension array for PfHRP2 and pLDH**
278 **detection**

279 **Optimization of standard curves for the detection of PfHRP2 and pLDH.** The
280 coupling conditions were optimized based on a concentration range of 10 to 100 ug/mL
281 of coupled HRP2 and pan-pLDH antibodies and testing with recombinant antigens as
282 well as plasma samples from *Pf* infected pregnant women, showing slightly higher MFI
283 values at 25 ug/mL (data not shown). A range of in-house biotinylated detection mAbs
284 was tested, and the optimal concentration was found to be 1 µg/ml for the detection of
285 both antigens (data not shown).

286 PfHRP2 type A, slightly higher MFI values were obtained for type A compared to types
287 B and C (See Additional file 2: Figure S1A), similarly to previously reported data
288 (24,25). PfHRP2 type A was selected as reference material. Recombinant *P. falciparum*
289 pLDH was detected down to lower concentrations compared to *Pv* pLDH, indicating
290 higher assay sensitivity for the detection of recombinant *P. falciparum* pLDH (Figure
291 1B). Similarly, the assay was able to detect lower concentrations of native *P. falciparum*
292 pLDH compared to *P. vivax* pLDH (See Additional file 2: Figure S1B). Additionally,
293 the detection of PfHRP2 and pLDH in assay buffer spiked with recombinant proteins,
294 cultured parasites or plasma samples yielded similar MFI values in singleplex and
295 multiplex (See Additional file 2: Figure S1C), with a clear correlation for both PfHRP2
296 (n = 25, r = 0.995; p < 0.001) and pLDH (n = 31, r = 0.992; p < 0.001), indicating no
297 cross-reactivity between PfHRP2 and pLDH components.

298 **Correspondence to International Units.** In the qSAT assay presented here, 1 IU
299 PfHRP2 corresponds to 23.5 pg PfHRP2, whereas 1 IU pLDH corresponds to 160 pg/ml
300 pLDH.

301 **Assay parameters**

302 **Limit of detection, limits of quantification and range.** The lower limit of detection
303 (LLOD) of the assay was determined to be 6.0, 56.1 and 1093.20 pg/ml for recombinant
304 PfHRP2 type A, *P. falciparum* pLDH and *P. vivax* pLDH respectively; and the lower
305 limit of the quantification (LLOQ) was estimated at 6.8 pg/ml for PfHRP2, 78.1 pg/ml
306 for *P. falciparum* pLDH and 1343.5 pg/ml for *P. vivax* pLDH. The ULOQ was found to
307 be 762.8 pg/ml, 17076.6 pg/ml and 187288.5 pg/ml for PfHRP2, *P. falciparum* pLDH
308 and *P. vivax* pLDH, respectively. The limits of detection for PfHRP2 types B and C
309 were 17.2 pg/ml and 15.8 pg/ml, respectively.

310 **Dilution linearity and accuracy.** The mean percent change in dilution-corrected
311 concentration between contiguous dilutions was 13.6 and 11.1% for PfHRP2 and *P.*
312 *falciparum* pLDH, respectively, as determined over 66 independent runs. These data are
313 within the acceptance criteria of +/- 20% (38). However, at concentrations close to the
314 ULOQ, the percent change showed an overestimation greater than 20% for both
315 PfHRP2 and *P. falciparum* pLDH (Table 2). The overall percent deviation between the
316 experimental concentration and the expected concentration for each serial dilution point
317 falling within or close to the analytical range was 19.6 and 16.4% for PfHRP2 and
318 pLDH, respectively. At concentrations falling at the LLOQ and the ULOQ, accuracy
319 decreased both for PfHRP2 and *P. falciparum* pLDH detection as shown in Table 2.

320 **Precision.** Intra-assay variation was 8.3% and 9.8% for PfHRP2 and pLDH,
321 respectively. The inter-assay %CV was 8.4% for the detection of PfHRP2 and 11.2%
322 for the detection of pLDH. For both antigens, intra-assay and inter-assay variation fell
323 within the acceptance criteria of 15% and of 20 % variation, respectively (30).

324 **PfHRP2 and pLDH recovery from dried blood spots**

325 To determine the loss of antigen when recovering PfHRP2 and pLDH from filter papers
326 as compared to same volumes of whole blood samples, DBS were prepared with whole
327 blood samples from Senegalese and Peruvian asymptomatic individuals (See Table 1).
328 Blood was eluted from DBS in assay buffer (See Additional file 1: Text4) and eluted
329 samples were assayed on the bead-suspension array along with the original whole blood
330 samples used to prepare the DBS. The geometric mean antigen concentration obtained
331 from DBS eluted product was 0.04 ng/ml (95% CI 0.03-0.07 ng/ml) for pHRP2 and
332 0.10 ng/ml (95% CI 0.06-0.16 ng/ml) for pLDH. These concentrations are 22.8 (n=38,
333 95% CI 15.6-33.5) and 59.7 (n=18, 95% CI 35.4-100.6) times lower than the
334 concentrations obtained in whole blood for PfHRP2 and pLDH, respectively (0.77
335 ng/ml (95% CI 0.37-1.61 ng/ml) for PfHRP2 and 5.77 ng/ml (95% CI 2.45-13.57
336 ng/ml) for pLDH), for identical blood volumes.

337 **Assay selectivity for the target antigens**

338 An important step in the development of the assay was to investigate whether it was
339 selective for the target antigens. Significant MFI signal for PfHRP2 and pLDH was
340 observed in *P. falciparum* positive samples (PfHRP2: Mean = 10195, SD = 12545;
341 pLDH: Mean = 9634, SD = 11765), whereas positive *P. vivax* samples (n=12) only
342 showed fluorescence signal for pLDH (Mean = 12960; SD = 3735), and not for PfHRP2
343 (Mean = 75.0, SD = 39.3) as expected (Figure 2). Five out of 71 and seven out of 738
344 negative samples by microscopy and PCR, respectively, showed MFI values above the
345 LLOQ for both PfHRP2 and pLDH, and four other *P. falciparum* positive samples by
346 microscopy and four *P. falciparum* positive samples by qPCR yielded greater MFI
347 values than the LLOQ for pLDH and PfHRP2 respectively. In addition, two *P.*
348 *falciparum* positive samples with *pfhrp2* deletion showed MFI values above the LLOQ.

349 Finally, all plasma samples (n=75) from Spanish malaria naïve pregnant women yielded
350 negligible fluorescence signals for both antigens (Figure 2).

351 **Correlation between antigen levels and parasite densities**

352 In samples positive for one or two antigens, the correlation between antigen
353 concentrations and parasite densities was investigated. Overall, a significant correlation
354 between PfHRP2 and parasite densities was found regardless of whether parasite
355 densities were quantified by qPCR (Spearman $r = 0.59$; $p < 0.0001$) or microscopy
356 (Spearman $r = 0.40$; $p < 0.0001$) (Figure 3). pLDH levels showed a higher correlation
357 with parasite densities compared to PfHRP2, both in samples for which densities were
358 determined by qPCR (Spearman $r = 0.75$; $p < 0.0001$) and by microscopy (Spearman $r =$
359 0.75 ; $p < 0.0001$) (Figure 3). The correlation between parasite densities and antigen
360 levels differed across the different sample sets analysed (See Additional file 3: Table
361 S1). Interestingly, the correlation between pLDH levels with parasite densities in whole
362 blood samples from Peru (Spearman $r = 0.76$; $p < 0.0001$) and Nigeria (Spearman $r =$
363 0.78 ; $p < 0.0001$) was very similar, whereas for PfHRP2, a better correlation with
364 parasite densities was found in samples from Nigeria (Spearman $r = 0.47$; $p < 0.0001$)
365 compared to samples from Peru (Spearman $r = 0.20$; $p = 0.0308$).

366

367 **DISCUSSION**

368 In the present study, we have established a quantitative suspension array based on
369 Luminex technology for the simultaneous detection and quantification of *P. falciparum*
370 HRP2 and *P. falciparum* and *P. vivax* pLDH, which allows to determine protein
371 concentrations as low as 6.0, 56.1 and 1042.7 pg/ml, respectively. Hence, the assay
372 provides increased sensitivity compared to commercially available ELISA kits which

373 have LODs of approximately 400 pg/ml and 1000 pg/ml for PfHRP2 and pLDH,
374 respectively (27,39). The assay shows good levels of dilution linearity, accuracy and
375 precision, and can be used to effectively and rapidly quantify malaria antigens in large
376 quantities of different biosamples.

377 The performance of the bead suspension array to quantify PfHRP2 and pLDH was
378 evaluated using reference recombinant proteins as well as cultured parasites, and in
379 different biofluids from malaria exposed and malaria naïve individuals. The assay is
380 selective for the target antigens and has an analytical range of 6.8 to 762.8 and of 78.1
381 to 17076.6 pg/ml for PfHRP2 and *P. falciparum* pLDH, respectively. Additionally, the
382 assay can also quantify *Pv* pLDH down to 1211.6 pg/ml. The assay analytical sensitivity
383 to detect PfHRP2 is comparable to that of a recently developed bead suspension assay
384 based on Luminex technology (25), as well as to other immunoassays that use different
385 technologies (20,28). This suggests that with the current technology available for the
386 quantification of PfHRP2 using antibodies, the lowest limit of detection achievable is in
387 the range of 0.5 to 10 pg/ml. The limit of detection for pLDH is more divergent across
388 assays, ranging from approximately 10 pg/ml (28) up to 4000 pg/ml (25), but in all
389 assays it is always higher than that for PfHRP2. This underpins the need to further
390 improve the sensitivity of pLDH-based diagnostics.

391 The bead suspension array described here can successfully be used as for detection and
392 quantification of PfHRP2 and pLDH in whole blood, eluted DBS and plasma or serum
393 samples. The concentration of eluted PfHRP2 from DBS to be equivalent to
394 approximately a 1:20 dilution from whole blood, similarly to previously reported data
395 (40). Differently, for pLDH we found that antigen concentration in eluted DBS
396 corresponds to a 1:60 whole blood dilution, which differs from previously published
397 data showing no differences in antigen recovery between PfHRP2 and pLDH (20).

398 However, such differences could be explained by the different extraction methodologies
399 and storage conditions used.

400 The quantification of PfHRP2 and pLDH is performed by interpolating MFI values to a
401 regression curve fitted from a calibration curve consisting of recombinant proteins
402 PfHRP2 type A and *P. falciparum* pLDH. Particularly for PfHRP2, the use of a single
403 recombinant protein as a reference material to quantify antigen levels in field samples
404 may provide an approximate estimate of the true concentration. PfHRP2 contains
405 sequences rich in histidine that form the epitopes targeted by the mAbs in RDTs (41),
406 which have been shown to be highly polymorphic in sequence composition of the
407 repeated motifs, as well as in overall length and number of repeated motifs between
408 different parasite strains (41). Baker *et al.* classified PfHRP2 as types A, B, or C
409 depending on the frequency of two epitope repeats (named type 2 and type 7) which
410 confer increased reactivity to mAbs in RDTs (41,42). According to this classification,
411 PfHRP2 Type A comprises the higher number of repeat types 2 and 7, followed by
412 PfHRP2 Type B, and finally PfHRP2 Type C. Our results on the detection of different
413 PfHRP2 types (See Additional file 2: Figure S1A) align with this data and resemble
414 recently published results (24,25).

415 We observed an overall positive significant correlation between antigen levels and
416 parasite densities similar to what previous studies have found (24), although the
417 correlation was different among the groups of samples analysed (See Additional file 3:
418 Table S1), probably because of the type of sample used for antigen quantification,
419 operational variations and sample storage. Of note, pLDH better correlated with parasite
420 densities compared to PfHRP2. This finding can be explained by the fact that PfHRP2,
421 differently from pLDH, is secreted to the blood stream and persists in circulation for
422 several days. In addition, we observed that the correlation between PfHRP2 and parasite

423 densities was lower in samples from Peru compared to samples from Nigeria, whereas
424 pLDH levels correlated very similarly to parasite densities in both groups of samples.
425 The high number of suspected *P. falciparum* positive samples with *pfhrp2* gene
426 deletions within the group of samples from Peru most probably explains this finding.

427 **CONCLUSIONS**

428 The quantitative suspension array technology presented here allows for a simultaneous
429 highly sensitive detection of the most commonly used target antigens in malaria RDTs.
430 The assay could be used as a tool to validate next generation RDTs, as well as to
431 estimate malaria burden in endemic areas and to evaluate the impact of malaria control
432 activities. Finally, this assay has the potential to be further upgraded by multiplexing the
433 detection and quantification of antibodies against parasite antigens that could serve as a
434 supplementary tool to study malaria transmission intensity, as well as the detection of
435 other infectious diseases antigens.

436 **Abbreviations**

437 α : anti; Ag: antigen; CI: confidence intervals; DBS: dried blood spot; EDC: 1-Ethyl-3-
438 [3-dimethylaminopropyl] carbodimide hydrochloride; ELISA: enzyme-linked
439 immunoabsorbent assay; GM: Geometric mean; IgG: immunoglobulin G; LLOD: lower
440 limit of detection; LLOQ: lower limit of quantification; LM: light microscopy; mAbs:
441 monoclonal antibodies; MFI: median fluorescence intensity; p/ μ l: parasites per
442 microliter; PCR: polymerase chain reaction; *Pf*: Plasmodium falciparum; pg/ml:
443 pictograms per millilitre; PfHRP2: Plasmodium falciparum histidine-rich protein 2;
444 PfHRP3: Plasmodium falciparum histidine-rich protein 3; pLDH: parasite lactate
445 dehydrogenase; *Pv*: Plasmodium vivax; qPCR: quantitative polymerase chain reaction;
446 qSAT: quantitative suspension array technology; RDT: rapid diagnostic test; RT: room

447 temperature; SD: standard deviation; Sulfo-NHS (N-hydroxysulfosuccinimide); ULOQ:
448 upper limit of quantification; μ l: microliter; 5PL: five parameters logistic; °C: degrees
449 Celsius; %CV: percent coefficient of variation; %DEV: percent deviation.

450 **Declarations**

451 **Authors' contributions**

452 AM and AC conceived and designed the study. CM, EM, ES and RG obtained the
453 plasma samples from Mozambican pregnant women. AV obtained the serum samples
454 from Colombian pregnant women. XD and SD were in charge of studies that allowed
455 collecting the whole blood samples from Senegalese and Peruvian asymptomatic
456 patients, and from Nigerian and Peruvian febrile patients, respectively. AJ and XMV
457 performed all laboratory experiments. AJ, AM and XMV performed the statistical
458 analyses and manuscript preparation. AC, AM, IG and XD provided overall study
459 supervision. All authors read and approved the final manuscript.

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463 University of Antioquia in Colombia, at University Cheikh Anta Diop in Senegal, at
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470 International Development (AECID). ISGlobal is a member of the CERCA Programme,
471 Generalitat de Catalunya.

472 **Competing interests**

473 The authors declare that they have no competing interests.

474 **Availability of data and material**

475 The datasets used and/or analysed during the current study are available from the
476 corresponding author on reasonable request.

477 **Consent for publication**

478 Not applicable.

479 **Ethics approval and consent to participate**

480 The Mozambican National Health and Bioethics Committee, the Medical Research
481 Center Ethics Committee at the Medicine Faculty of Universidad de Antioquia and the
482 Hospital Clinic of Barcelona Ethics Committee approved the use of non-identifiable
483 plasma and serum samples in the current study. Written informed consent was obtained
484 from all participants.

485 The Senegal National Ethics Committee (Comité National d’Ethique pour la Recherche
486 en Santé) reviewed and approved on 15 January 2015 the study protocol associated with
487 the collection of whole blood specimens from consenting asymptomatic adults in
488 Senegal to support the development and evaluation of new assays for the detection of
489 malaria infections (Protocol SEN14/74). Written informed consent was obtained from
490 all participants.

491 The Universidad Peruana Cayetano Heredia Institutional Review Board (Comité
492 Institucional de Ética) reviewed and approved on 10 March 2015 the study protocol
493 associated with the collection of whole blood specimens from consenting asymptomatic
494 adults in Peru to support the development and evaluation of new assays for the detection
495 of malaria infections (Protocol 100-02-15). Written informed consent was obtained
496 from all participants.

497 The study protocol for the evaluation of a multiplex fever diagnostic test was submitted
498 for ethics approval in October 2016 and December 2016 in Peru and Nigeria,
499 respectively, and approvals were obtained in November 2016 in Peru, and January 2017
500 in Nigeria. Informed consent was obtained from all participants or by legal guardians in
501 cases of underage participants.

502 The institutional review board at the Universidad Peruana Cayetano Heredia (Lima,
503 Peru) approved the study protocol the specimen collection campaign organized in April
504 2016 in the area of Iquitos (Peru).

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656 **ADDITIONAL FILES**

657 **Additional file 1.**

658 Format: .docx format.

659 Title of data: Supplementary Materials and Methods

660 Data: Includes “Text S1. Biotinylation of detection mAbs”, “Text S2. Bead suspension
661 array procedure”, “Text S4. Singleplex versus Multiplex testing”, and “Text S4.
662 Preparation and extraction of proteins from dried blood spots”

663 **Additional file 2.**

664 Format: .pdf format

665 Title of data: Supplementary figure 1.

666 Description of data:

667 **Figure S1. Assay optimization.** (A) Serial dilutions of recombinant PfHRP2 types A, B
668 and C were assayed to determine the lowest concentration at which each antigen is
669 detected; (B) *P. falciparum* Benin I and Borneo and *P. vivax* field isolates were assayed
670 in a serial dilution fashion to assess differences between the analytical sensitivity for *P.*
671 *falciparum* and *P. vivax* pLDH; (C) PfHRP2 and pLDH positive samples (plasma,
672 cultured field isolates and recombinant proteins) were assayed in singleplex (X axes)
673 and multiplex (Y axes).

674 **Additional file 3.**

675 Format: .docx format

676 Title of data: Supplementary Tables 1 and 2.

677 Description of data:

678 Table S1. Correlation between antigen levels and parasite densities for each group of
679 clinical samples analysed.

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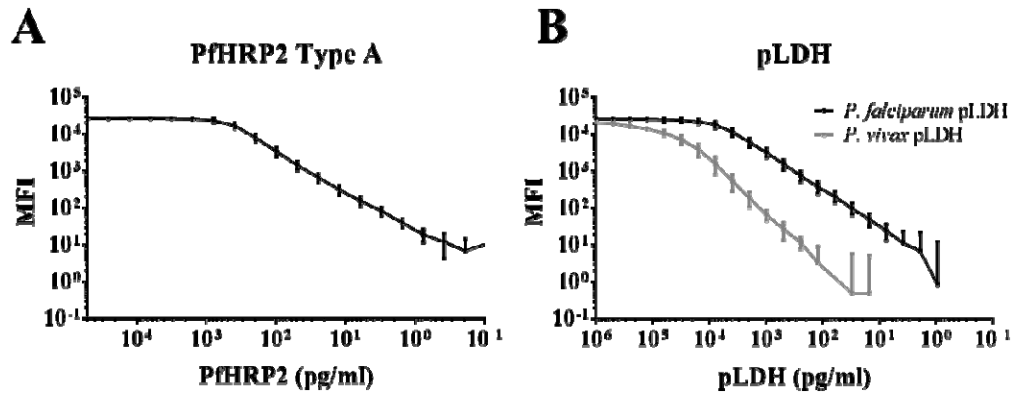
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687 **FIGURES AND TABLES**

688 **Figure 1. Calibration curves to detect PfHRP2, Pf pLDH and Pv pLDH.**
689 Recombinant *P. falciparum* (*Pf*) HRP2 type A (A) and *Pf* (B, back line) and *P. vivax*
690 (*Pv*) (B, grey line) pLDH were serially diluted to investigate the assay analytical range.
691 Error bars show the standard deviation of the mean from 66 independent reads for
692 PfHRP2 type A and *Pf* pLDH, and 12 reads for *Pv* pLDH. X axis: MFI value after



693 subtraction of the background.

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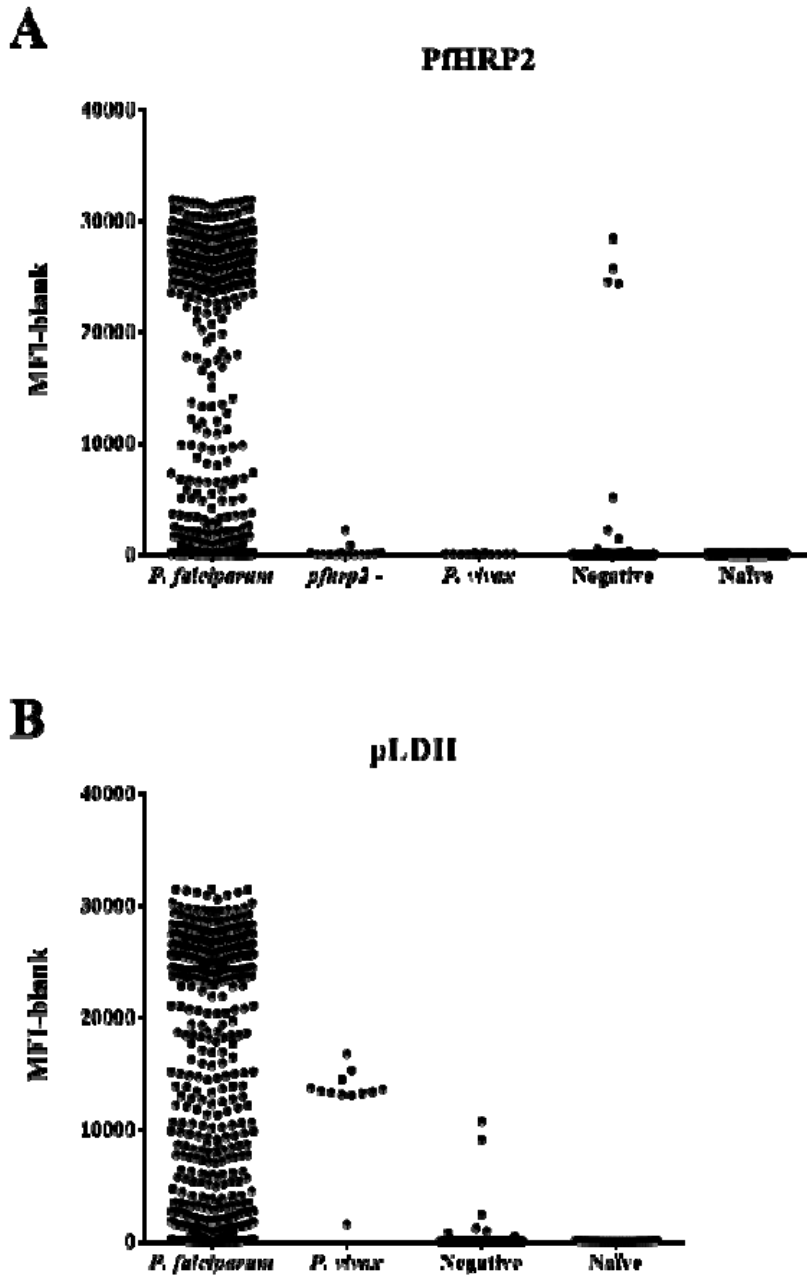
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708 **Figure 2. The quantitative bead suspension array is selective for PfHRP2 and**
709 **pLDH.** Median fluorescence intensity with blank subtracted for PfHRP2 (A) and pLDH
710 (B) for *P. falciparum* positive samples (n = 1098), *P. falciparum* with *hrp2* gene
711 deletions (n=16), *P. vivax* positive samples (n = 12), *Plasmodium* negative samples,
712 and samples from naïve individuals (n =75). *pfhrp2* -: *Plasmodium falciparum* with
713 *hrp2* gene deletion.

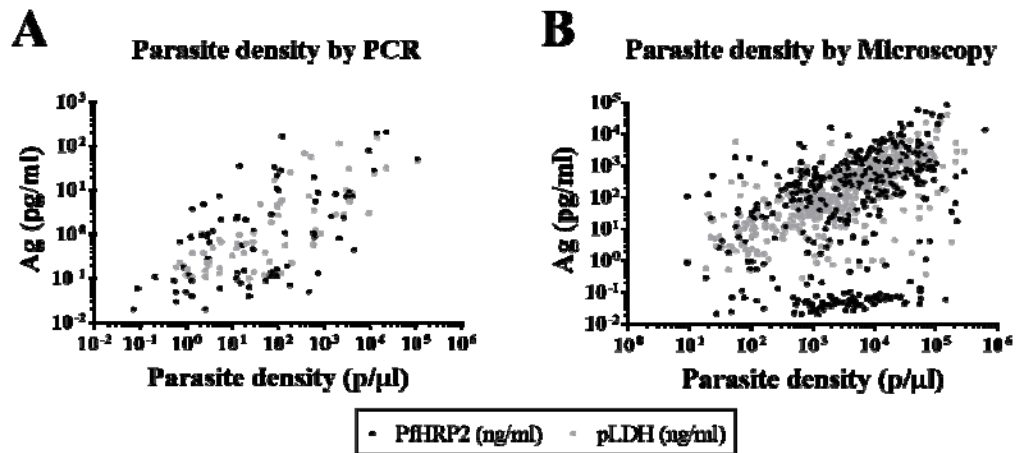


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717 **Figure 3. Antigen levels correlate with parasite densities.** Correlation of parasite
718 densities (p/ μ l) with PfHRP2 and pLDH concentration (pg/ml) in *P. falciparum* positive
719 samples by PCR (A), and by microscopy (B).



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737 **Table 1. Clinical samples tested on the qSAT assay.**

Samples (n)	Type of sample	Origin	Sampling period	Population	Reference assay	<i>P. falciparum</i> positive (n)	GM parasite density (p/μl) (95% CI)	Others
765	Plasma	Mozambique	2010 - 2012	Pregnant women	qPCR on DBS	59	127 (58.4-276.3)	
103	Serum	Colombia	2005 - 2007	Pregnant women	Microscopy	32	3901 (2059-7391)	
110	EDTA whole blood	Senegal	NA	Adults	Nested PCR and qPCR on WB*	55	4.9 (2.6-9.4)	
16	EDTA whole blood	Peru	April - August 2015	Adults	Nested PCR and qPCR on WB*	16	52.7 (19.1-146.0)	<i>pfhrp2</i> deleted
639	EDTA whole blood	Nigeria	April - August 2017	Aged ≥5 years	Microscopy and PCR**	639	4713 (3530-6292)	
323	EDTA whole blood	Peru	March - October 2017	Aged ≥5 years	Microscopy and PCR**	323	1719 (1328-2225)	
75	Plasma	Spain	2010	Pregnant women	NA	NA	NA	

GM: Geometric mean.

* Nested PCR was used for species determination and qPCR for parasite density quantification. *P. falciparum* samples were positive for both methods.

** PCR was used for species determination and microscopy for parasite density quantification. Geometric mean parasite densities is calculated on the basis of microscopy positive samples (n = 247/639 in Nigeria, and 191/323 in Peru).

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Sample	<i>Pf</i> pLDH			PfHRP2		
	Expected concentration (pg/ml)	% Change	% Deviation	Expected concentration (pg/ml)	% Change	% Deviation
1	15625	41.6	24.2	781.3	32.8	23.4
2	7812.5	18.3	17.4	390.6	18.5	10.8
3	3906.3	0.9	17.1	195.3	6.8	10.2
4	1953.1	2	10.7	97.7	0	8.5
5	976.6	5.6	10.4	48.8	5.2	10.1
6	488.3	4.2	14.2	24.4	14.7	19
7	244.1	5.5	14.9	12.2	12.7	27.9
8	122.1	10.4	22.2	6.1	18.5	47
Overall	-	11.1	16.4	-	13.6	19.6

740 **Table 2. Dilution linearity and accuracy of qSAT assay.**

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