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Detection of *Plasmodium falciparum* malaria in 1 h using a simplified enzyme-linked immunosorbent assay

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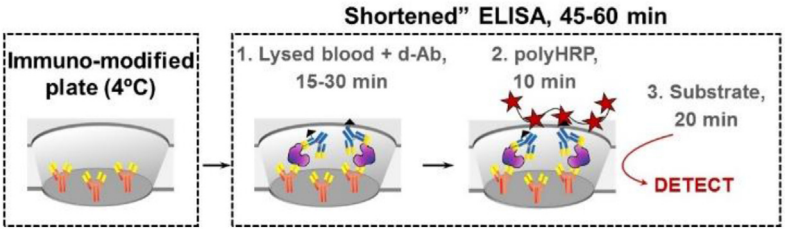
HIGHLIGHTS

- Simplified ELISA based in signal amplifier, antibody selection and sample pretreatment.
- Quantitative malaria diagnosis in whole blood samples in 45–60 min.
- Performance comparable to PCR, but faster, cheaper and less technically demanding.
- Method compatible with high throughput testing and population screening.

GRAPHICAL ABSTRACT



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ABSTRACT

Malaria is a parasitic disease caused by protists of the genus *Plasmodium*, which are transmitted to humans through the bite of infected female *Anopheles* mosquitoes. Analytical methodologies and efficient drugs exist for the early detection and treatment of malaria, and yet this disease continues infecting millions of people and claiming several hundred thousand lives each year. One of the reasons behind this failure to control the disease is that the standard method for malaria diagnosis, microscopy, is time-consuming and requires trained personnel. Alternatively, rapid diagnostic tests, which have become common for point-of-care testing thanks to their simplicity of use, tend to be insufficiently sensitive and reliable, and PCR, which is sensitive, is too complex and expensive for massive population screening.

In this work, we report a sensitive simplified ELISA for the quantitation of *Plasmodium falciparum* lactate dehydrogenase (Pf-LDH), which is capable of detecting malaria in 45–60 min. Assay development was founded in the selection of high-performance antibodies, implementation of a poly-horseradish peroxidase (polyHRP) signal amplifier, and optimization of whole-blood sample pre-treatment. The simplified ELISA achieved limits of detection (LOD) and quantification (LOQ) of 0.11 ng mL^{-1} and

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0.37 ng mL^{-1}, respectively, in lysed whole blood, and an LOD comparable to that of PCR in *Plasmodium in vitro* cultures (0.67 and 1.33 parasites μL^{-1} for ELISA and PCR, respectively). Accordingly, the developed immunoassay represents a simple and effective diagnostic tool for *P. falciparum* malaria, with a time-to-result of <60 min and sensitivity similar to the reference PCR, but easier to implement in low-resource settings.

1. Introduction

Malaria is a parasitic disease caused by protists of the genus *Plasmodium*, which are transmitted to humans by the bite of infected female *Anopheles* mosquitoes [1]. Several species of *Plasmodium* can cause infections in humans. Among them, *P. falciparum* is the most frequent (accounting for over 99% of malaria cases in Africa) and the one that produces the most serious clinical symptoms. Billions of US$ have been invested in control and elimination policies to accomplish the World Health Organization (WHO) objective of eradicating malaria [2]. Nevertheless, the disease incidence rate, which had declined globally from 71 to 57 cases per 1000 among the population at risk between 2010 and 2014, has remained stable since then. According to the WHO, in 2018 there were 228 million cases of malaria worldwide that caused the death of some 405,000 people (62% of them children aged under 5 years) [2]. The WHO estimates that, using current tools, there will still be 11 million cases of malaria in Africa in 2050, impeding to set a target date for malaria eradication [3].

The availability of efficient diagnostic tools, able to provide rapid and objective quantification of the amount of parasite present in the patient's blood, even at very low titers, will be crucial to achieve malaria eradication [4,5]. First, because prompt personalised diagnosis and treatment are the most effective way to prevent mild malaria cases from developing into severe disease and death. Second, because the correct detection of non-malarial infections with similar symptoms avoids unnecessary treatments, reducing side effects and the selection of drug-resistant parasites. And third, because the identification of infected individuals that remain asymptomatic or display low parasitaemias is crucial to control the parasite transmission dynamics. Currently, malaria is primarily diagnosed by three types of tools: light microscopy, rapid diagnostic tests (RDTs), and nucleic acid amplification [6,7]. Light microscopy performed by an expert is considered the gold standard [7,8]. Microscopy allows both counting the parasites present in a blood sample and identifying the parasite species. Nevertheless, it implies subjective result interpretation, requires a long time (>1 h per sample) and does not allow detecting very low parasitaemias (also known as submicroscopic malaria), which makes it inefficient for high-throughput population screening. Alternatively, RDTs are devices easy to use by unqualified personnel that have assay times spanning 15−30 min and a cost of € 1−5 [9]. Most commercial malaria RDTs are lateral flow devices that work similarly to pregnancy tests and detect circulating parasite histidine-rich protein II (HRP2), which is produced only by *P. falciparum*. The lack of reactivity against other *Plasmodium* species and the identification of deletions in the *pfhrp2* and *pfhrp3* genes in *P. falciparum* parasites, which may prevent detection by HRP2-based RDTs [2,10], are encouraging the use of biomarkers expressed by most *Plasmodium* species, such as pan-*Plasmodium* lactate dehydrogenase (pLDH) and aldolase [11,12]. Regrettably, RDTs display variable performance and sensitivity depending on the provider, production batch, patient's parasitaemia, parasite species, or temperature and humidity conditions during product storage [13]. In addition, the available RDTs are qualitative and insufficiently sensitive and reliable to identify submicroscopic parasitaemias. Finally, molecular techniques, such as the polymerase chain reaction (PCR), allow the detection of parasite genetic material in blood [6]. In addition to being highly sensitive and specific, PCR can be fully automated and allows differentiating between species as well. However, it is a technique that requires experienced personnel, precise technical equipment and sensitive reagents, which makes it difficult to implement in low-resource settings, such as in sub-Saharan Africa where most malaria cases occur.

Although a few reports proposed the use of biosensors and microfluidic systems as powerful alternatives for the detection of *Plasmodium*, most examples were not validated in clinical samples and/or entailed equipment and handling hardly compatible with high-throughput screening [14–17]. In contrast, Enzyme-Linked Immunosorbent Assay (ELISA) is a classical methodology extensively used in diagnostic laboratories that is prone to automation, miniaturization and high-throughput testing [18]. Atchade and coworkers showed in 2013 that a pLDH ELISA was a valid tool for population screening in endemic areas, detecting in 2.5 h down to 1 parasite μL^{-1} in cultured *Plasmodium*-infected red blood cells (RBCs; equivalent to 80 pg mL^{-1} of pLDH) [12]. Attempts have been made to improve assay performance since then. For instance, Jang et al. optimized a multiplexed ELISA using Quansys Q-Plex technology that achieved quantification of HRP2, pLDH and *P. vivax* LDH (Pv-LDH) in PCR-confirmed infections in >3.5 h, with LODs of 2.3, 47.8, and 75.1 pg mL^{-1}, respectively [19]. Lee et al. described a microfluidic-based ELISA that, using Optimiser™ microplates with a spiral 3D channel, provided results in <90 min, detecting *P. falciparum* LDH (Pf-LDH) down to 1 pg μL^{-1} in spiked human serum [20]. In a different approach, Martiáñez-Vendrell and coworkers optimized a Luminex fluorescent assay for the multiplexed detection of HRP2, Pf-LDH and Pv-LDH (LODs of 6, 56 and 1093 pg mL^{-1}) that took 2.5 h plus an overnight incubation [21].

The objective of this work was to produce a short and simple ELISA for the detection of malaria infection in whole blood samples, able to provide *Plasmodium* quantification below 1 parasite mL^{-1} in <60 min with little user intervention, which was not afforded in the previous examples. To achieve this goal, (i) a battery of antibodies was tested to select the best-performing pair, (ii) a high-performance ELISA was optimized using a poly-horseradish peroxidase (polyHRP) signal amplifier, and (iii) whole-blood sample pre-treatment was investigated to increase the amount of free Pf-LDH. The performance of the simplified ELISA developed was finally assessed in *Plasmodium* cultures and in samples from patients.

2. Experimental section

2.1. Reagents and biocomponents

Four pairs of capture/detection monoclonal antibodies against pLDH were tested: (1) references C01834 M/C01835 M from Meridian Bioscience (Memphis, TE, USA); (2) A63640297P/63610297P

and (3) self-pairing A63611314P from BiosPacific (Emeryville, CA, USA); and (4) 10–1336/10-1335 from Fitzgerald (Acton, MA, USA). All detection antibodies (d-Ab) were biotinylated (Supporting Information).

Recombinant Pf-LDH (A3005) was obtained from CTK Biotech (San Diego, CA, USA). polyHRP (Ref. 21140, Thermo Scientific; Madrid, Spain) consisted of streptavidin-conjugated polymerized HRP. Streptavidin-HRP (Ref. DY998) and reagent diluent concentrate 2 (Ref. DY995; equivalent to 10 × phosphate-buffered saline (PBS) with 10% bovine serum albumin, BSA) were from R&D Systems (Minneapolis, MN, USA).

Tween-20, 3,3′,5,5′-tetramethylbenzidine liquid substrate (TMB; Ref. T4444), 1 M sulfuric acid, sodium chloride, and 1 M imidazole buffer solution were obtained from Sigma-Aldrich (Madrid, Spain). 3-(N,N-dimethylmyristylammonium) propanesulfonate (SB14, 97%), Triton X-100, tris base and tris (hydroxymethyl) aminomethane hydrochloride (tris-HCl) were provided by Fisher Scientific (Madrid, Spain). Human RBC lysis buffer (Ref. J62990; containing 150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.2–7.4) was from Alfa Aesar (now Fisher Scientific). PBS tablets (10 mM sodium phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4) were from Gibco (Life Technologies; Madrid, Spain). For washing, PBS was supplemented with 0.05% Tween-20 (PBS-T). For the blocking and incubation steps, PBS was supplemented with 1% BSA (PBS-BSA) or with 1% BSA plus 0.05% Tween-20 (PBS-T-BSA). The 96-well high-binding microtiter plates were purchased from Corning (Corning Life Sciences; Amsterdam, The Netherlands).

2.2. Detection Pf-LDH by ELISA

Unless otherwise stated, all incubations were performed with 100 μL of solution per well, inside an incubator thermostated at 35 °C, and were followed by three washes with PBS-T (200 μL well^{-1}), which were carried out manually.

2.2.1. Pf-LDH detection by classical ELISA

A classical multi-step ELISA was employed for the characterization and selection of the Ab pairs and as the reference assay (Fig. 1a and b). Briefly, microtiter plates were modified for 1 h with capture antibody (c-Ab; 2.5 μg mL^{-1} in PBS) and were blocked with PBS-BSA for 1 h. The plates were then incubated for 1 h with Pf-LDH (0.016–100 ng mL^{-1} in PBS-BSA), for 1 h with detection antibody (d-Ab; 100 or 200 ng mL^{-1} in PBS-BSA) and for 20 min with streptavidin-HRP (1:200 in PBS-BSA). After four more washes, the plates were incubated for 20 min with TMB before stopping the reaction with sulfuric acid (50 μL well^{-1}) and measuring the absorbance at 450 nm using a Sunrise plate reader (Tecan; Männedorf, Switzerland).

2.2.2. Pf-LDH detection by shortened ELISA

Upon optimization, the shortened ELISA consisted of the modification of the microtiter plate with c-Ab as detailed above and blocking with PBS-T-BSA, followed by a 15-min ($ELISA_{15min}$) or a 30-min ($ELISA_{30min}$) simultaneous incubation with Pf-LDH and d-Ab (200 ng mL^{-1}, in PBS-T-BSA) (Fig. 1a,c). The plate was then washed, incubated for 10 min with polyHRP (1:10,000 in PBS-T-BSA), washed four times and detected as described before.

2.3. Collection, pre-processing and storage of clinical samples

Whole blood samples were collected from patients infected with malaria attending to Vall d'Hebron University Hospital (VHUH) between 2018 and 2019. Malaria infections were confirmed by microscopic blood examination and/or by PCR [22]. The Ethics Committee of VHUH approved the study (PR(AG)30/2018) and informed consent was signed by all patients.

Peripheral blood was drawn in EDTA and heparin collection tubes. Plasma was immediately separated by centrifugation at 3000 rpm for 10–15 min at 20 °C, and was aliquoted and stored at −80 °C. Whole blood was diluted 1:1 with lysis buffer (LB), incubated for 5 min at room temperature, and finally aliquoted and stored at −80 °C. Five LBs were compared here: LB1 included 50 mM NaCl, 300 mM K_3PO_4, 250 mM imidazole and 1% Triton X-100; LB2 was LB1 without imidazole; LB3 consisted of 200 mM tris buffer supplemented with 55 mM SB3-14 (a zwitterionic surfactant) and 1% Triton X-100; LB4 was a ready-to-use commercial buffer containing 150 mM ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM EDTA (Alfa Aesar J62990); and LB5 was MilliQ water (osmotic shock).

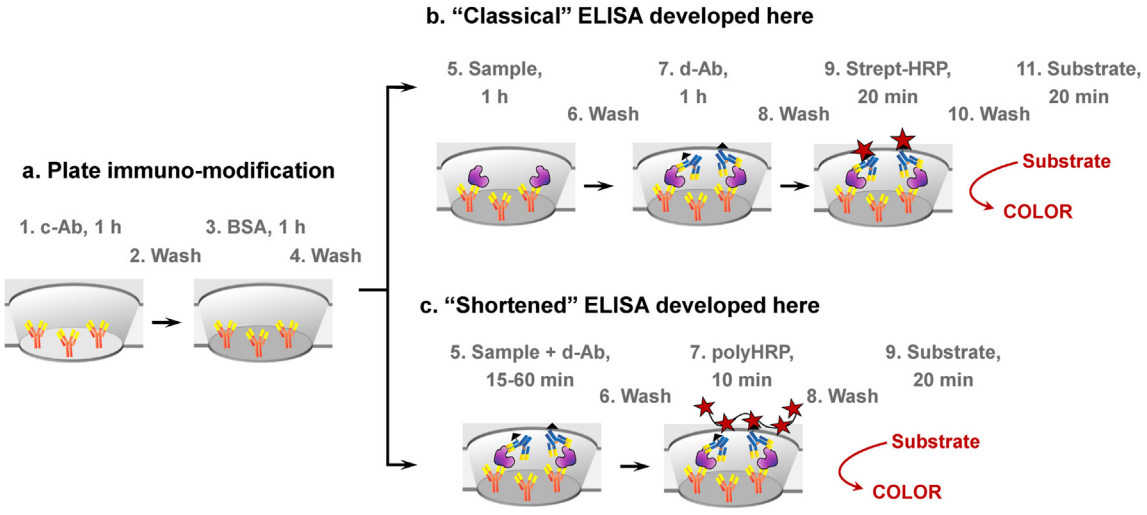

Fig. 1. Schematic representation of the classical and the shortened ELISAs developed here. In both cases, the microtiter plate was modified with c-Ab and blocked to prevent non-specific adsorption (a). However, while the classical ELISA continued with a series of incubations that lasted for nearly 3 h (b), the shortened assay was carried out in 45–90 min (c).

2.4. P. falciparum culture in vitro

P. falciparum was grown at 37 °C with human RBCs following standard protocols (Supporting Information) [23]. For culture maintenance, parasitaemias were kept below 5% by dilution with fresh RBCs. For standard assays, the culture was maintained under stirring for 3–5 days, when parasitaemia was adjusted with >90% of synchronized parasites. *Plasmodium* culture parasitaemias ranging from 0.0058 to 1.5% (37% haematocrit) were then obtained by serial dilution of the initial culture with fresh RBCs and medium. Parasitaemia of the initial culture was determined by flow cytometry [24] and the different culture dilutions were characterized by real-time PCR (RT-PCR) to obtain the corresponding cycle threshold values (Ct; see the Supporting Information for more details) [25]. For the study with the $ELISA_{30min}$, cultured samples were treated with LB1 as described above.

2.5. Data analysis

Calibration plots were fit to a second order polynomic using Excel. The assay linear range was selected as the section of the plot for which signal displayed linear correlation against analyte concentration with highest R^2. The assay limits of detection (LOD) and quantification (LOQ) were calculated as the average of the blanks (without Pf-LDH) plus 3 and 10 times their standard deviation (SD), respectively. The sensitivity corresponded to the slope of the assay linear range. The signal-to-noise ratio (S/N) was calculated by dividing the signal obtained for each Pf-LDH concentration by the signal obtained for the blanks. The variability was calculated in terms of coefficient of variation (%CV = (SD/mean) × 100). Samples were tested in triplicate per microtiter plate and each experiment was repeated at least twice.

3. Results and discussion

3.1. Antibody selection by classical ELISA

Four different pairs of Abs were tested that, according to the providers, were specific for pLDH and did not bind human LDH or other molecular components potentially present in clinical samples. Fig. 1a depicts schematically the classical ELISA protocol used, which was re-optimized for each Ab pair separately (Figs. S1 and S2). On top of the time required for plate immuno-modification and blocking (2 h), the classical ELISA took nearly 3 h.

As illustrated in Fig. 2a–b, the classical ELISA detected in all cases Pf-LDH within the concentration range 0.78–100 ng mL^{-1}. The Ab pairs tested generated very similar results, except for the self-pairing Ab provided by BiosPacific (Ref. A63611314, used as both c-Ab and d-Ab), which displayed narrower linear range, faintly higher sensitivity (slope) and lower LOD/LOQ (Table 1).

3.2. Optimization of a simplified ELISA

Several authors have reported the use of polyHRP as a signal amplifier to improve ELISA sensitivity [26–28]. The increase in signal provided by polyHRP has been exploited alternatively to reduce the assay incubation times, producing short and simple immunoassays and immunosensors [29–31]. A similar approach was explored here (Fig. 1). For this, the two consecutive immunocaptures performed in the classical ELISA (taking 1 h each) were substituted by a single simultaneous incubation of the immuno-modified plate with Pf-LDH and d-Ab, which was extended for 15, 30 or 60 min ($ELISA_{15min}$, $ELISA_{30min}$ and $ELISA_{60min}$). At the same time, the 20-min incubation with streptavidin-HRP was replaced by a 10-min incubation with polyHRP. As a result, the assay duration was reduced from about 3 h to 45–90 min.

Interestingly, the shortened assays displayed similar or better figures of merit than the classical ELISA for the 4 pairs of Ab tested (Table 1; Figs. S3 and S4). For instance, in most cases the linear range of the shortened assays shifted towards lower Pf-LDH concentrations compared to the classical ELISA and the LODs/LOQs were lower. Although longer single-step immunocaptures produced slightly better results, three of the Ab pairs (Meridian C01834 M/C01835 M and BiosPacific A63640297P/63610297P and self-pairing A63611314) still displayed better figures of merit in the $ELISA_{15min}$ than in the 3-h classical ELISA. Furthermore, for two of the Ab pairs (A63611314P/A63611314P and C01834 M/C01835 M), the shortened ELISAs worked similarly at room temperature and at 35 °C (Fig. S5).

Of the 4 pairs of Ab tested, Meridian C01834 M/C01835 M were selected because they produced the best results in the shortened assay formats studied, displaying in general lower LOD/LOQ and higher signals and S/N than the other Ab pairs (Fig. 2c and d; Fig. S6). For this Ab pair, all the shortened assays showed better performance than the classical ELISA, with intra- and inter-assay variability below 10% and 15%, respectively (Fig. 2e and f; Fig. S7). Furthermore, upon optimization of the blocking and storage conditions, the immuno-modified plates were stable for at least 4 months at 4 °C without losing performance (Fig. S8-11).

3.3. Optimization of whole blood sample pre-treatment

In patients infected with malaria the majority of *Plasmodium* parasites are found inside the RBCs. Consequently, RBC lysis should release intracellular parasites, increasing the amount of detectable Pf-LDH. Five lysis buffers (LB1-5) were compared here (see section 2.3).

For this study, whole blood obtained from 3 malaria patients was mixed 1:1 with each LB and was incubated for 5 min. The level of haemolysis was then determined microscopically and, after centrifugation to pellet the remaining RBCs, by visual inspection of the pelleted cells and by studying spectrophotometrically the supernatants.

Massive haemolysis only occurred after treatment with LB containing Triton X-100, a non-ionic detergent commonly used for cell membrane solubilization at room temperature. The degree of haemolysis was proportional to the concentration of Triton X-100, which induced haemoglobin release and decrease in the size of the pellet (Fig. 3a). Reddish supernatants were detectable by the naked eye only for samples treated with concentrations of Triton above 0.06% and a minimum of 1% of Triton X-100 was needed to suppress pellet formation. Microscopic inspection of the samples corroborated that LBs containing Triton produced more efficient cell lysis than the other LBs (Fig. 3b–g, Fig. S12). Of the 3 Triton-containing LBs, LB1 generated the highest level of haemolysis measured spectrophotometrically in the supernatants of the three samples (Fig. S13).

Triton-based lysis also enhanced Pf-LDH detection by ELISA (Figs. S14a–b). Fig. 3h shows that the amount of Pf-LDH detected by $ELISA_{30min}$ in samples lysed with LB1 was in average 4 times higher than the concentrations observed in untreated samples, which confirmed that the selected lysis treatment enhanced Pf-LDH availability. On the other hand, the anticoagulant employed in the blood collection tubes had little effect on Pf-LDH quantification using $ELISA_{30min}$ (Fig. S14c).

3.4. Detection of Pf-LDH by shortened ELISA in spiked and clinical samples

The shortened ELISAs developed were next tested in different Pf-LDH-spiked matrices, calibrated in a battery of artificial samples

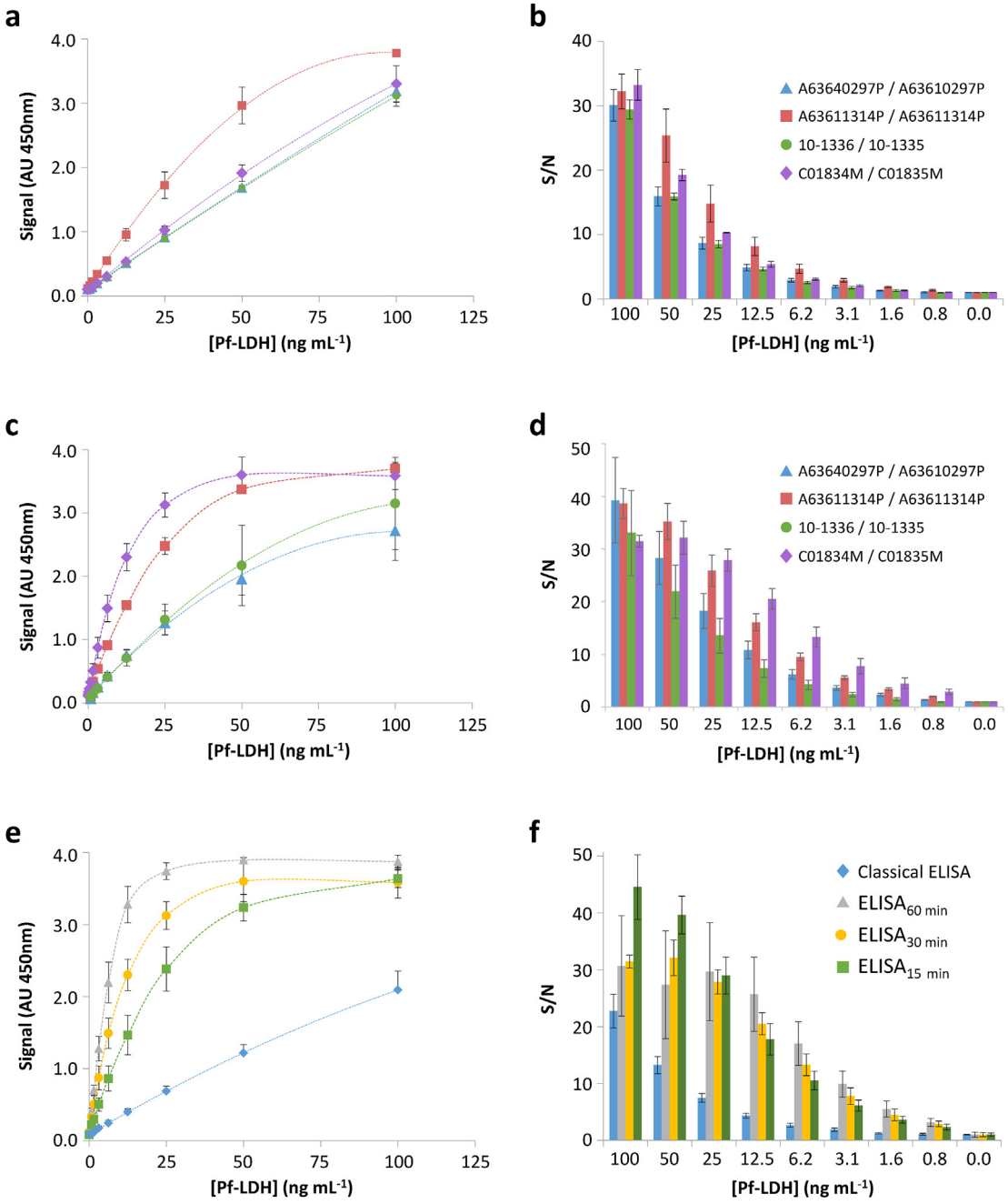

Fig. 2. ELISA optimization and Ab selection for Pf-LDH detection. (a–d) Calibration curves and S/N ratios obtained by classical ELISA (a,b) and by $ELISA_{30min}$ (c,d) for the 4 Ab pairs tested. (e,f) Comparative performance of the different ELISA formats when carried out using the best-performing Ab pair selected here (C01834 M/C01835 M). Data shown correspond to the averages of no less than 3 independent replicates and the error bars display the corresponding standard deviations.

displaying controlled *P. falciparum* parasitaemias, and finally applied to the analysis of 10 whole blood samples from malaria-infected patients.

3.4.1. *Interference of sample matrix in assay performance*

An interference study was carried out in order to determine the performance of the ELISA30min in complex sample matrices. For this, plasma, whole blood and lysed whole blood from healthy individuals (not presenting Pf-LDH) were diluted to different extents, spiked with increasing concentrations of recombinant Pf-LDH, and analyzed by $ELISA_{30min}$. Plasma did not interfere with the assay, even at a 1:10 dilution, generating signals and S/N comparable to those of PBS-T-BSA (Fig. 4a–b). On the other hand, blood produced contradictory results. Detection of Pf-LDH-spiked untreated whole blood produced higher signals and S/N than the test performed with PBS-T-BSA (Fig. 4c–d). This effect was not observed in the negative controls without Pf-LDH, suggesting that it was not caused by Ab crossreaction with human LDH (Fig. S15), and was attributed to higher stability of the Ab in blood. In contrast, when Pf-LDH was spiked in lysed blood (diluted 1:10), in which erythrocytes did not contain intracellular Pf-LDH and thus lysis was not expected to increase its concentration, the signals generated decreased (Fig. 4e–f). This was more evident for samples lysed with LB1 than with LB4 (which did not contain Triton), suggesting that the signal

Table 1
Summary of the analytical characteristics of the classical and simplified ELISAs for the different pairs of Ab tested. Whole assay duration includes the time needed to perform the corresponding washing steps and spectrophotometric measurement.

Pair of antibodies	Assay type	Incubation (min)			Whole assay duration	Linear range (ng.mL^{-1})	LOD (ng.mL^{-1})	LOQ (ng mL^{-1})	Sensitivity (AU·mL·ng^{-1})
		Pf-LDH	bd-Ab	HRP					
C01834 M/C01835 M	Classical ELISA	60	60	20	3 h	1.56–50.00	0.74	1.96	358
	$ELISA_{60min}$	60		10	90 min	0.20–3.12	0.13	0.35	3253
	$ELISA_{30min}$	30		10	55 min	0.20–6.25	0.11	0.37	1697
	$ELISA_{15min}$	15		10	45 min	0.39–12.50	0.18	0.58	1341
A63640297P/A63610297P	Classical ELISA	60	60	20	3 h	1.56–100.0	1.00	3.09	307
	$ELISA_{60min}$	60		10	90 min	0.39–12.50	0.11	0.50	902
	$ELISA_{30min}$	30		10	55 min	0.39–12.50	0.10	0.51	536
	$ELISA_{15min}$	15		10	45 min	0.78–25.00	0.37	0.95	518
A63641314P/A63641314P	Classical ELISA	60	60	20	3 h	1.56–25.00	0.48	1.56	638
	$ELISA_{60min}$	60		10	90 min	0.39–6.25	0.25	0.50	1357
	$ELISA_{30min}$	30		10	55 min	0.39–12.50	0.19	0.56	1147
	$ELISA_{15min}$	15		10	45 min	0.39–12.50	0.16	0.91	869
10-1336/10-1335	Classical ELISA	60	60	20	3 h	0.78–50.00	0.8	1.73	341
	$ELISA_{60min}$	60		10	90 min	0.78–25.00	0.30	0.83	890
	$ELISA_{30min}$	30		10	55 min	1.56–25.00	0.87	2.10	498
	$ELISA_{15min}$	15		10	45 min	1.56–50.00	0.98	2.59	279

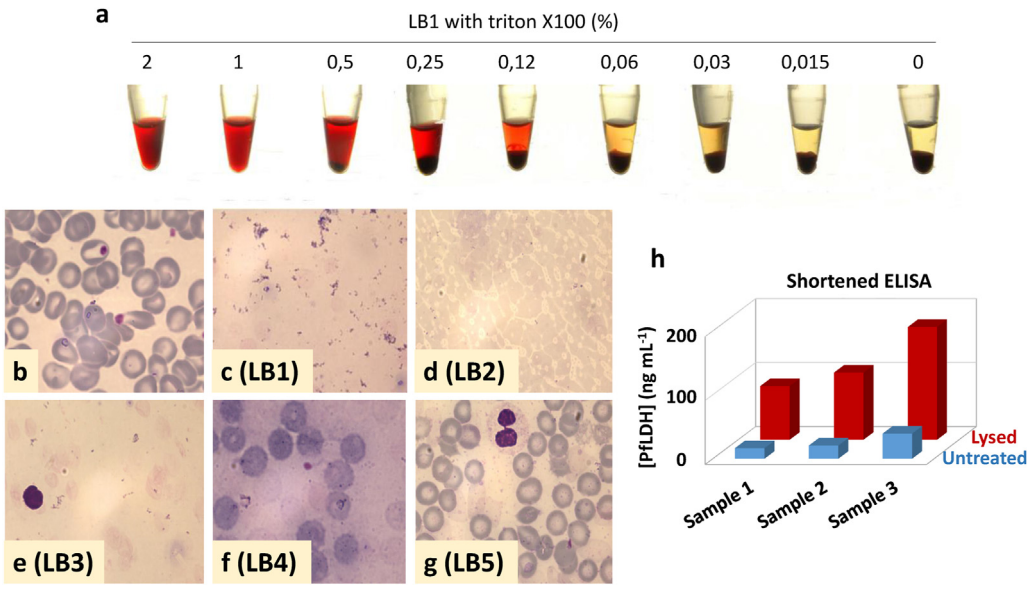

Fig. 3. Optimization of RBC lysis. (a) Effect of the concentration of Triton X-100 on RBC lysis. (b–g) Microscopic observation of whole blood from a patient infected with malaria before (b) or after lysis for 5 min with LB1-5 (c–g). (h) Pf-LDH quantification by $ELISA_{30min}$ (C01834 M/C01835 M Ab pair) in whole blood from 3 malaria patients before and after lysis (5 min, LB1).

decrease could be caused by the release of proteases in lysed samples, Triton-mediated partial protein denaturation, and/or Triton-enhanced washing of poorly bound reagents. Nevertheless, Pf-LDH was detected in the whole range of concentrations studied in lysed blood, suggesting that the $ELISA_{30min}$ developed was useful for the study of clinical samples.

3.4.2. Assay calibration in synthetic samples of controlled parasitaemia

The $ELISA_{30min}$ was next calibrated by detecting Pf-LDH in samples displaying a wide range of known *P. falciparum* parasitaemias. This was accomplished by studying cultured RBCs (45% haematocrit), infected *in vitro* with *P. falciparum* at parasitaemias spanning between 0.000014 and 1.5% (equivalent to 0.67–87209 parasites μL^{-1}). For their study, samples were lysed for 5 min to release intraerythrocytic parasites, diluted serially with PBS-T-BSA, and finally analyzed in triplicate using the $ELISA_{30min}$. All samples were analyzed in parallel (previous to lysis) using the reference RT-PCR technique.

The developed $ELISA_{30min}$ detected Pf-LDH in the whole battery of cultured RBC samples, providing concentrations of Pf-LDH that correlated linearly with the theoretical parasite loads (Fig. 5a) and with RT-PCR quantification (Fig. 5b). The results confirmed that, in the case of *Plasmodium*-infected samples, which displayed intra-erythrocytic parasites, Pf-LDH detection improved if RBCs were lysed (Fig. S16a). Although LB1 and LB2 seemed equally effective for the detection of Pf-LDH in medium to high parasitaemias, the samples displaying less than 20 parasites μL^{-1} could be detected only after LB1 lysis (Fig. S16b). In addition, while in LB1-lysed samples the assay detected on average 194 fg of Pf-LDH per parasite (with a range spanning 120–326 fg per parasite), 127 fg per parasite were detected in LB2-lysed samples (range spanning 64–184 fg per parasite), and just 60 fg of Pf-LDH per parasite could be detected in non-lysed samples (ranging 24–88 fg per parasite). Accordingly, 1 parasite μL^{-1} was equivalent to a concentration of Pf-LDH between 0.06 and 0.2 ng mL^{-1}, depending on sample pre-treatment, which is consistent with the values reported by other

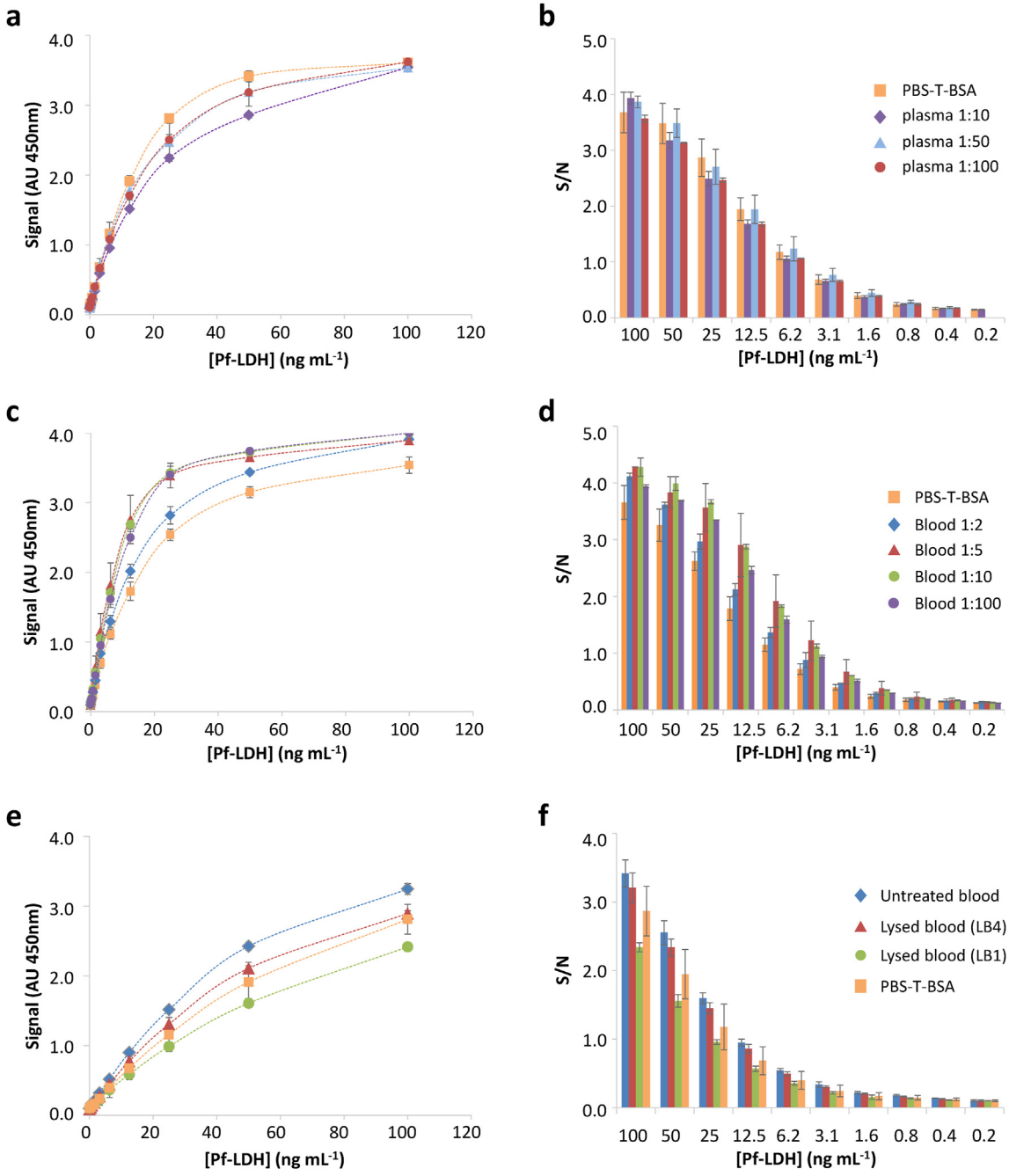

Fig. 4. Sample matrix interference in the ELISA$_{30min}$. Signals (left) and S/N (right) obtained for different concentrations of Pf-LDH spiked in sample matrices of increasing complexity: (a–b) plasma diluted 1:10–1:100; (c–d) whole blood (EDTA) diluted 1:2–1:100; (e–f) lysed whole blood diluted 1:10 (LB1 and LB4). Data shown correspond to the averages and standard deviations obtained from 3 independent replicates.

authors in *P. falciparum* cultures. For instance, Atchade et al. estimated that 1 parasite μL^{-1} corresponded to 0.08 ng mL^{-1} of recombinant pLDH using a commercial ELISA, and a magnetic bead ELISA reported by Markwalter and co-workers detected 1.3 pM of pLDH in 1 parasite μL^{-1} samples (which would be equivalent to 0.044–0.088 ng mL^{-1} of recombinant pLDH) [11,12].

The new method displayed an LOQ of around 1.3 parasites μL^{-1}, which was comparable to that of the reference RT-PCR (parasitaemias >1.33 parasites μL^{-1}). This value is below the threshold recommended by the WHO for the identification of low parasite density malaria cases (200 parasites μL^{-1}) [32]. It was thus concluded that the ELISA$_{30min}$ developed in this work was sensitive enough for the study of clinical samples. Furthermore, it was easier, cheaper and faster to carry out than RT-PCR.

3.4.3. Analysis of clinical samples

Table 2 summarizes the concentrations of Pf-LDH measured after different pretreatments of the samples from 4 patients exhibiting *P. falciparum* parasitaemias that ranged from submicroscopic to over 3%. As anticipated, sample pre-treatment had a profound effect on Pf-LDH quantification in the clinical samples as well. In general, medium to high parasitaemias (from 0.2% to >3%) could be detected in plasma and untreated whole blood, which exhibited comparable concentrations of circulating Pf-LDH.

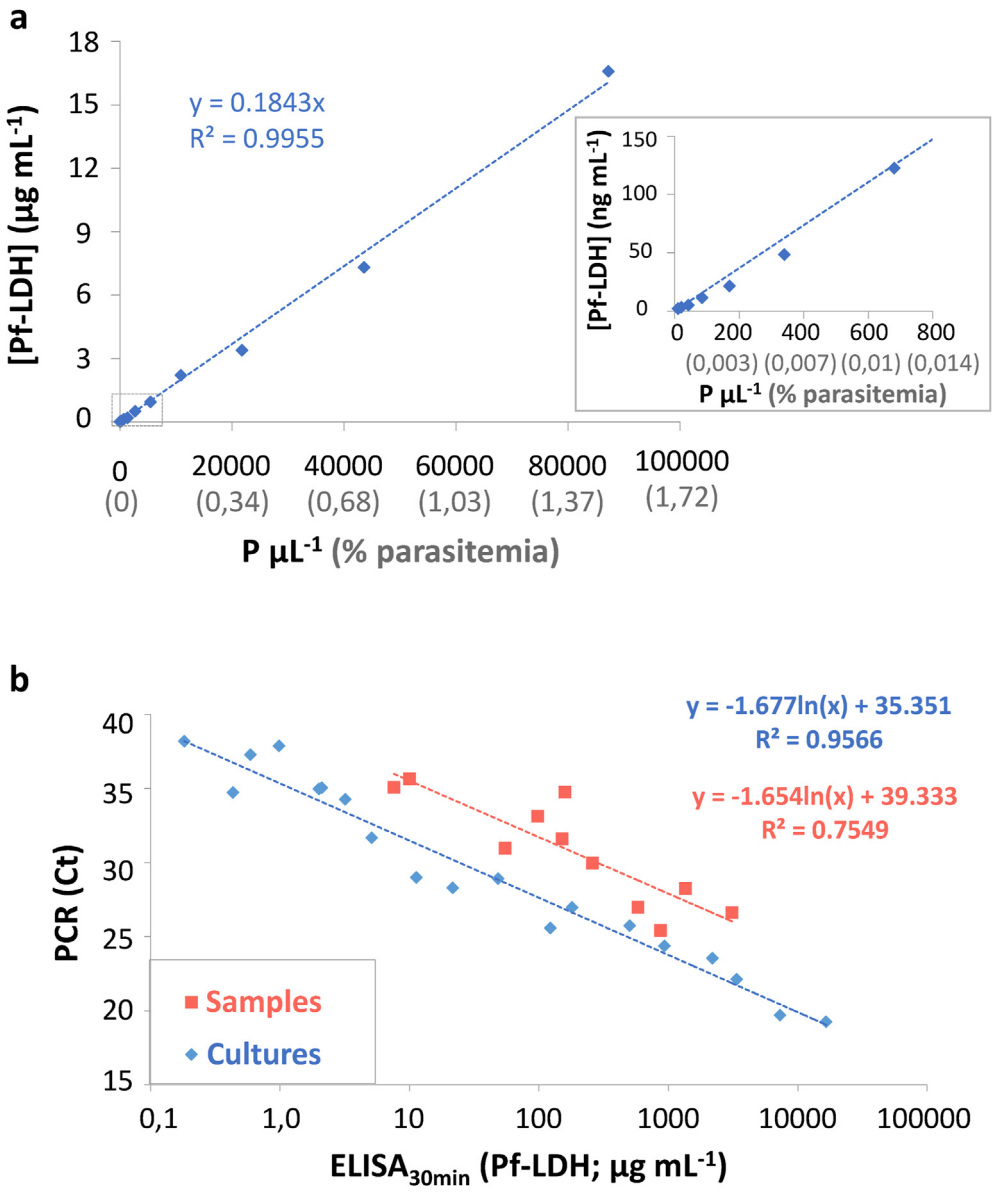

Fig. 5. Detection of Pf-LDH in cultured RBCs infected *in vitro* with *P. falciparum* and in lysed whole blood samples from malaria patients. (a) Concentrations of Pf-LDH detected using the $ELISA_{30min}$ in cultures displaying increasing parasite concentrations (P μL^{-1}). In brackets, the corresponding % parasitaemias estimated by RT-PCR. (Inset) Amplification of the boxed section. (b) Correlation between the concentrations of Pf-LDH detected by $ELISA_{30min}$ and parasite detection by RT-PCR (cycle threshod value, Ct). For their study by $ELISA_{30min}$, samples were lysed and diluted serially from 1:4 to 1:4000 (cultures), or 1:10 and 1:100 (samples from patients). The signals obtained by $ELISA_{30min}$ were then interpolated in a calibrate run in parallel and the concentrations detected in diluted samples were multiplied by the corresponding dilution factor to estimate Pf-LDH concentration in the pre-diluted samples (shown in the graphs).

However, blood lysis increased the amount of free Pf-LDH, especially when using LB1. This effect seemed proportional to parasitaemia, which could be related to the parasite exponential growth (the concentration of Pf-LDH was 3–5 and 10–70 times higher in lysed samples for 0.2 and 3% parasitaemias, respectively). In contrast, low parasitaemias (0.01%) could be detected only in LB1-lysed whole blood. This confirmed that it was necessary to analyze lysed whole blood to detect those samples displaying very low concentrations of Pf-LDH, such as sub-microscopic malaria, and that LB1 was the best-performing lysis buffer.

On the other hand, most infections could be detected if samples were analyzed in parallel after 1:10 and 1:100 dilution. The matrix effect observed in 1:10 lysed blood produced concentration sub-estimates of nearly 50% (Table 2), but allowed detection of submicroscopic malaria. In contrast, the quantification of mid and high Pf-LDH concentrations was more robust and reproducible in 1:100 blood.

Finally, the $ELISA_{30min}$ and $ELISA_{15min}$ were used to study 14 clinical restrospective samples, 10 from malaria patiens infected with *P. falciparum* and 4 from patients with infections other than malaria. All samples had been lysed with LB1, aliquoted and stored at −80 °C. Pf-LDH was detected successfully in the 10 malaria samples, revealing predilution concentrations spanning 7–3100 ng mL^{-1}, but in none of the negative controls. The concentrations of Pf-LDH obtained with the two ELISAs correlated well (Fig. S17a) and were consistent with the results obtained by RT-PCR (Fig. 5b). Clinical samples exhibited higher amounts of Pf-LDH than cultures with comparable RT-PCR results, presumably because they had been lysed by mixing 1:2 with LB1, which interfered in the RT-PCR, an effect that was not studied additionally here. Besides,

Table 2
Pf-LDH quantification by $ELISA_{30min}$ in plasma and whole blood (EDTA) from 4 patients infected with *Plasmodium falciparum* and displaying parasitaemias ranging from submicroscopic to 3% (*SM* stands for submicroscopic and numbers in square brackets correspond to patient parasitemia). Samples were analyzed in triplicate, either directly or after lysis with LB1 or LB4, and after dilution to different extents depending on the sample (the table shows the final dilution, including lysis as well). *<LOQ* and *OVER* indicate that the signals registered were below the assay LOQ and above saturation, respectively; *nd* stands for not determined. Bold numbers indicate concentration estimates in pre-diluted samples (once dilution correction factor was applied) and the corresponding numbers in brackets show the coefficient of variability (% CV).

	Concentration of Pf-LDH (ng mL-1)			
SAMPLE	S1 [SM]	S2 [0.01%]	S3 [0.2%]	S4 [3.1%]
Plasma, 1:2	<LOQ	nd	nd	nd
Plasma, 1:10	<LOQ	nd	72 (9,69%)	25 (2.6%)
Plasma, 1:100	Nd	<LOQ	91 (8,68%)	40 (1,08%)
Plasma, 1:500	Nd	<LOQ	nd	85 (2,26%)
Blood, 1:2	<LOQ	nd	nd	nd
Blood, 1:10	<LOQ	nd	40 (9,76%)	17 (4,53%)
Blood, 1:100	Nd	<LOQ	64 (12,91%)	52 (5,88%)
Blood, 1:500	Nd	<LOQ	nd	80 (4,46%)
Blood + LB4, 1:4	<LOQ	nd	nd	nd
Blood + LB4, 1:10	<LOQ	nd	43 (11,79%)	105 (6,40%)
Blood + LB4, 1:100	Nd	47 (9,52%)	80 (15,88%)	278 (8,24%)
Blood + LB4, 1:500	Nd	<LOQ	nd	170 (10,70%)
Blood + LB1, 1:4	1.7 (11,29%)	nd	nd	nd
Blood + LB1, 1:10	3.1 (12,70%)	nd	200 (7,11%)	OVER
Blood + LB1, 1:100	Nd	136 (1,13%)	462 (13,1%)	2947 (2,57%)
Blood + LB1, 1:500	Nd	215 (2,51%)	nd	2050 (4,18%)

$ELISA_{30min}$ and $ELISA_{15min}$ granted detection of a submicroscopic malaria that had been missed by microscopy (Fig. S17b), which confirmed that the simplified ELISA developed here was efficient enough to detect low *P. falciparum* parasitaemias.

4. Conclusions

Of the three methods currently employed for malaria diagnosis, microscopy, RDTs and RT-PCR, none is sensitive, simple and efficient enough to provide detection of low malaria parasitaemias with little user intervention. Here, a simplified ELISA assay has been developed, which was shorter and simpler to carry out than classical ELISA, but displayed performance comparable to RT-PCR. A larger number of clinical samples will have to be tested in the future to confirm the reliability of this method. However, the current results suggest that the assay developed could provide Pf-LDH-based diagnosis of low *P. falciparum* malaria parasitaemias, including submicroscopic cases, which is not afforded by microscopy, the current gold standard. Compared to RDTs, which use to be qualitative rather than quantitative, the simplified ELISA provided quantitative detection of Pf-LDH over the whole assay range. In addition, the assay displayed LOD/LOQ comparable to those exhibited by RT-PCR, but results were provided faster (in just 60 min, instead of >4 h) and with less technical requirements. It is worth noting that, although the assay was carried manually here, ELISA-based techniques can be automatized for high-throughput sample processing, which suggests that the simplified ELISA reported here could be of help in mass screening programs as well. Furthermore, these results demonstrate that it is possible to produce last-generation ELISA assays without more technical requirements and cost than classical ELISA, but significantly faster.

Credit Author Statement

Eva Baldrich and Erica de la Serna conceived the method. Erica de la Serna carried most of the experimental work, analysed the data and wrote the first manuscript draft. Kevin Arias-Alpízar, Livia Neves Borgheti-Cardoso, Ana Sanchez-Cano, and Miriam Ramírez contributed to assay optimization and validation. Adrián Sánchez-Montalvá, Pau Bosch-Nicolau, Fernando Salvador, and Israel Molina recruited patients and obtained samples for method validation. Elena Sulleiro and Francesc Zarzuela characterized patient samples using reference methods. Xavier Fernàndez-Busquets, Adrián Sánchez-Montalvá and Eva Baldrich coordinated the work and produced the final manuscript. All authors participated in data interpretation and manuscript revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338254.

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