Malaria quantitative POC testing using magnetic particles, a paper microfluidic device and a hand-held fluorescence reader

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Abstract

A point-of-care (POC) device is reported for highly sensitive and selective detection of *Plasmodium falciparum* lactate dehydrogenase (Pf-LDH), a biomarker of malaria infection, based on a single-step magneto-immunoassay, a single-use microfluidic paper device and a customized hand-held fluorescence reader. The single-step magneto-immunoassay consists in a single 5-min incubation of immuno-modified magnetic particles (c-MAb-MPs), biotinylated detection antibody (bd-MAb), and an enzymatic signal amplifier (Poly-HRP). After on-chip MP concentration and washing, signal generation is achieved by adding a fluorescent enzymatic substrate (QuantaRed). Fluorescence signal is measured using a low-cost customized, portable, and sensible fluorescent detector. The POC affords quantitative Pf-LDH detection in <20 min, with a detection limit of 0.92 ng·mL^{-1} (equivalent to 4.6 parasites µL^{-1}). Furthermore, Pf-LDH quantitation in clinical samples correlates with that provided by the reference ELISA, is more sensitive than a commercial rapid diagnostic test (RDT) and entails little user intervention. These results show that fluorescent paper-based microfluidic devices can be exploited to simplify magneto-immunoassay handling, taking this type of test closer to the requirements of POC testing.

Keywords

Immuno-modified magnetic particles; paper-based diagnostic device; low-cost assay automation; portable fluorimeter; point-of-care testing; malaria quantitative diagnosis.

1 Introduction

Malaria continues to be a serious public health problem and one of the most harmful parasitic infectious diseases worldwide. According to the World Health Organization (WHO), 3.3 billion people live in risk areas. In 2020, malaria caused 241 million new cases and 627000 deaths, 67% of these among children under 5 years (WHO, 2021). Malaria is a vector-borne disease caused by a protozoa of the genus *Plasmodium*, which is transmitted to humans by the bite of infected female mosquitos of the genus *Anopheles* (Cowman et al., 2016). Of the *Plasmodium* species that infect humans, *P. falciparum* is the most common and lethal, and accounted for 94% and 94.4% of total cases and deaths, respectively, reported in 2019 (WHO, 2020). Access to rapid and accurate diagnosis is key in the efforts to eradicate this disease. An appropriate diagnostic is crucial to discern malaria from diseases with similar clinical profiles and to personalize treatment, minimizing the risk of parasite resistance to antimalarial drugs (Zarei, 2018). However, this is challenging in remote locations and low-resource countries, due to the lack of equipment, expertise, and infrastructure.

The gold standard to identify malaria is still microscopy, which allows detection down to 5-10 parasites per µL of blood ($p \cdot \mu L^{-1}$) in the hands of an expert. (Mouatcho and Dean Goldring, 2013; Wu et al., 2015). Although inexpensive, this technique relies on well-trained microscopists, subjective result interpretation, long analysis time (2-4 h) and it is not sensible enough to detect submicroscopic malaria. Molecular techniques, such as the polymerase chain reaction (PCR), achieve limits of detection (LOD) down to 1 $p \cdot \mu L^{-1}$, but require long analysis time, experienced personnel and high operational costs.

In recent years, there has been growing interest in developing point-of-care (POC) analytical methodologies capable to perform fast and cheap diagnosis as close as possible to the patient (Heidt et al., 2020). Well-known examples are dipsticks, lateral flow rapid tests (RDTs), microfluidic devices, and biosensors (Qin et al., 2021; Rei Yan et al., 2020; Wang et al., 2021). Among them, paper-based devices outstand by their low cost, ease of use, and portability. These commonly exploit passive capillarity to transport the sample and reagents towards a detection zone, where colour generation allows result interpretation by the naked eye or using hand-held readers (Williams and Drennan, 2022). However, inhomogeneous color distribution on the test strip, interpretation variability between users, or insufficient sensitivity are issues reported commonly (Rei Yan et al., 2020).

Paper-based fluorescent assays have been explored as an alternative to overcome the limitations of colorimetric detection. One of the first examples of malaria detection was demonstrated by Carrilho *et al*., who replaced the conventional microtiter plates by 96-

and 384-microzone patterned paper plates, detecting either absorbance or fluorescence using a commercial reader (Carrilho et al., 2009). More recently, Geldert *et al*. developed a molybdenum disulfide (MoS_2) nanosheet-based aptasensor, detecting pan *Plasmodium* lactate dehydrogenase (pLDH) by fluorescence resonance energy transfer (FRET) (Geldert et al., 2017). Several teams employed MPs to detect *Plasmodium* antigens, often claiming faster detection, lower LODs and/or less interference from complex sample matrices than when using flat surfaces (Table S-1). However, most of these examples entailed multi-step assays carried entirely in tubes, in which MPs are submitted to series of incubations and washes using magnets, which requires user training. Singh *et al*. incorporated the use of paper to carry the fluorescent detection of a magneto-immunoassay. They used two MP-bound aptamers to capture in parallel pLDH and *P. falciparum* glutamate dehydrogenase (Pf-GDH) (Singh et al., 2019). An absorbent wick was then used to detect the enzymatic activity of pLDH/Pf-GDH, coupled to conversion of resazurin to resorufin (a pink fluorescent dye). The result was interpreted either colorimetrically in an instrument-free format, or fluorescently using a spectrophotometer, achieving qualitative and quantitative results, respectively. Ruiz-Vega *et al*. went a step farther, and used a paper screen-printed electrode to carry on-chip sample filtration, MP concentration and washing, and Pf-LDH electrochemical detection, showing that magneto-immunoassays can be partially automated using low-cost paper devices (Ruiz-Vega et al., 2020). Although electrochemical transduction is ideal for POC testing thanks to the potential cheapness, robustness and portability of the measurement equipment, miniaturized fluorescence readers have been recently fabricated using inexpensive LEDs and photodiodes which should be compatible with POC testing in low-resource settings (Alonso et al., 2020; Yao et al., 2012). Furthermore, while electrochemistry requires the use of electrodes, fluorescence can be measured in the dark on any surface or material not displaying auto-florescence.

Herein, we report a paper-based fluorescent magneto-immunoassay for detection of *P. falciparum* malaria. The assay consists of a single 5-min incubation of the lysed whole blood sample with a cocktail of reagents. This mixture is then directly pipetted in a single-piece paper-based device, fabricated using a low-cost craft cutter. Here, MP washing, magnetic concentration and fluorescent detection are accomplished on-chip with little user intervention. As we show, the system provides semi-quantitative detection when interpreted visually and quantitative detection using a customized portable fluorimeter, with limits of detection of 1.56 ng mL^{-1} and 0.9 ng mL^{-1} (equivalent to 4.5 $p{\cdot}\mu L^{-1}$), respectively. Furthermore, the analysis of patient whole blood samples took <20 min, providing Pf-LDH quantification comparable to the reference ELISA and better performance than a commercial RDT detecting pLDH.

2 Material and Methods

2.1 Reagents and biocomponents

Anti-pLDH capture and detection monoclonal antibodies (c-MAb and d-Mab, Refs. C01834M and C01835M) were from Meridian Bioscience (Memphis, TE, USA). The d-MAb was biotinylated to produce bd-MAb (Supporting Information). Recombinant Pf-LDH (Ref.A3005) was provided by CTK Biotech (San Diego, USA). Carboxylic acid MPs (MyOne, 1 µm diameter, Ref. 65011), Streptavidin Poly-HRP (Ref. 21140), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and QuantaRed Enhanced Chemifluorescent HRP Substrate Kit (Ref. 15159) were obtained from Thermo Fisher (Waltham, USA). MPs were modified with c-MAb to produce c-MAb-MPs (Fig. S-1). Bovine serum albumin (BSA), Triton X-100, Tween 20, 2-(N-morpholino)ethanesulfonic acid hydrate (MES), and 3,3′,5,5′-Tetramethylbenzidine Liquid Substrate System (Supersensitive) for ELISA (TMB; Ref. T4444) were from Sigma-Aldrich (Madrid, Spain). Reagent Diluent (ref. DY995, 10×RD, equivalent to 10x PBS, 10% BSA) was from R&D Systems Europe (Abingdon, UK). Phosphate-buffered saline tablets (PBS; pH 7.4) were obtained from Gibco (Life Technologies, Madrid, Spain). For washing, PBS was supplemented with 0.05% of Tween (PBS-T). The incubation buffer was 10×RD diluted 1:10 with MiliQ water and supplemented with Tween 0.05% (PBST-$BSA_{1\%}$). Standard 17 and CF5 membranes were obtained from GE Healthcare (Germany, Refs. 17114594 and 29008181). Colourless and black 96-well microtiter plates were purchased from Corning Life Sciences (Amsterdam, The Netherlands).

2.2 Whole blood samples

Whole blood samples were obtained over 2018-2019 from control individuals and from patients with acute malaria infection confirmed by microscopy, ELISA (Fig. S-2) and/or PCR. The study (PR(AG)30/2018) was approved by the Ethics Committee of Vall d'Hebron University Hospital and informed consent was signed by all patients.

Peripheral blood was collected in heparin collection tubes before the administration of any antimalarial drug. Whole blood was diluted 1:1 with lysis buffer (50 mM KH_2PO_4, 300 mM NaCl, 0.25 M imidazole, 1% Triton X-100), incubated for 5 min at room temperature, and aliquoted for storage at -80°C.

2.3 Pf-LDH magneto-immunodetection

Unless otherwise stated, MP were incubated in Eppendorf tubes at 24°C in a thermoshaker (Thermal Shake lite, VWR, Barcelona, Spain). Paper devices were incubated at room temperature under static conditions.

2.3.1 Single-step magneto-immunoassay carried in tubes

The single-step magneto-immunoassay was based in a previous development (Fig. S-3) (Sánchez-Cano et al., 2021). Briefly, before their utilization, c-MAb-MPs were washed twice with PBS and were resuspended in PBST-$BSA_{1\%}$ to 5 mg mL^{-1}. Pf-LDH (sample) was then stirred for 5 min at 1500 rpm, in 100 µL of PBST-$BSA_{1\%}$ with 4 µL of c-MAb-MPs, 5.5 µL of bd-MAb and 2.3 µL of Poly-HRP (final concentration of 75 ng mL^{-1} for bd-MAb and 50 ng mL^{-1} for Poly-HRP). For assay colorimetric detection, c-MAb-MPs were washed twice with 150 µL of PBS-T, were resuspended in 100 µL of TMB and were stirred for 20 min at 1500 rpm in the dark. MPs were concentrated, the supernatant was transferred to the wells of a 96-well plate, 50 µL of 1 M sulphuric acid were added, and absorbance was measured at 450 nm using a Sunrise plate reader (Tecan, Switzerland). For fluorescent detection, c-MAb-MPs were washed twice with 150 µL of PBS-T and once with PBS. c-MAb-MPs were then incubated with 100 µL of QuantaRed at 1500 rpm for 5 min. The supernatant was transferred to a black 96-well plate, 10 µL of stop solution were added and fluorescence was measured using a FLUOstar® Omega plate reader (BMG Labtech, Germany; 544 nm for excitation and 590 nm for emission).

2.3.2 Single-step magneto-immunoassay carried in a paper device

For assay on-chip handling and fluorescent detection, the 5-min immunocapture was performed in tubes as in section 2.3.1. This mixture was then transferred to the distal end of the washing pad of a paper device (Fig. 1). When it had been absorbed, 500-µL of PBS-T were added to the washing reservoir. This pushed the mixture to the MP concentration section, where a magnet retained the MPs while unbound reagents flowed towards the terminal absorption pad. Finally, 50 µL of QuantaRed were added to the bottom of the washing pad and the device was incubated for 5 min in the dark. Fluorescence detection was achieved using a home-made portable fluorimeter.

2.4 Fabrication of the paper-based sensor

The system developed here consists of two main components, a disposable paper sensor and a reusable magnetic holder (Fig. 1a). The single-piece paper sensor was designed using Silhouette Studio® version 4.4.476 and cut on Standard 17 membrane using a low-cost Silhouette Cameo 3 craft plotter (Silhouette America, Utah, USA; Fig. 1b). This sensor contained four distinguishable sections. One extreme displayed a washing reservoir, produced with a segment of a pipette tip and attached using a ring of Blu-Tack adhesive, to dispense the washing buffer. Next was a funnel-shaped washing pad (22 mm x 9.5 mm), where the mixture of reagents and lysed-blood sample were transferred. This section directed the reagents towards the MP concentration zone, providing efficient MP washing under flow conditions and subsequent magnetic

concentration. The third section provided a lecture zone for the fluorescent readout. Finally, the sensor displayed a circular end, which sat under an absorbent pad, 26x16 mm, made of CF5 using a guillotine. This pad provided flow driving and waste storage. For preventing biocomponent non-specific binding, paper sensors were blocked for 15 min at room temperature in PBS-T, BSA 5% (PBST-$BSA_{5\%}$), followed by two consecutive washes with PBS-T for 3 min each. Finally, the devices were dried for 30 min at 37°C. Paper sensors were stored in a ziplock pouch until used.

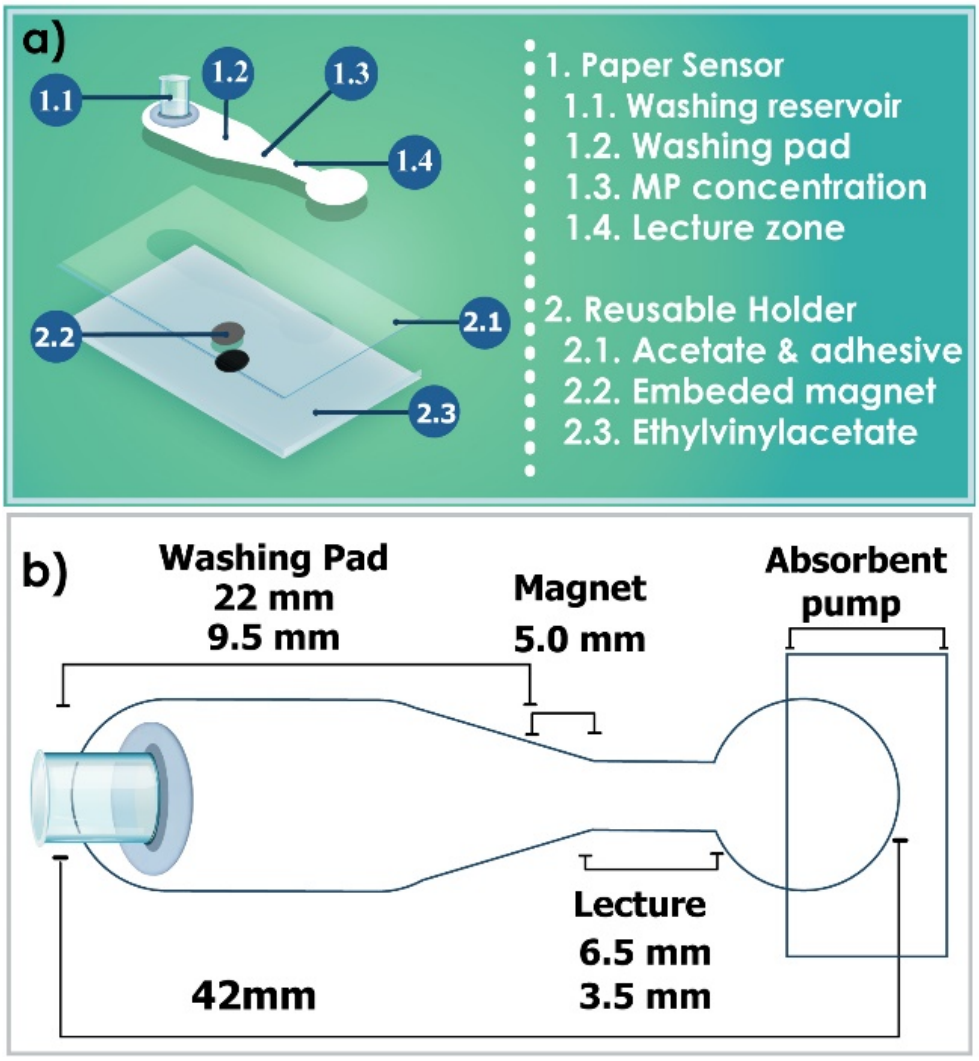

Figure 1. a) Schematic representation of the detection system developed here, including the disposable sensor (top) and the reusable magnetic holder (bottom). b) Dimensions of the single-piece paper device.

On the other hand, the reusable magnetic holder was made of ethylene-vinyl acetate (EVA; obtained from a local store). A hole crafted with a biopsy punch allocated a neodymium magnet, 1 mm thick and 5 mm in diameter. Two layers of acetate, cut with the Silhouette Cameo 3, were fixed on top using double-sided adhesive. While the first

one kept the magnet in place and prevented contact with the MPs, the second displayed an opening to accommodate the paper sensor and guarantee alignment with the magnet.

2.5 Portable fluorimeter

The portable fluorimeter used for paper-based magneto-immunoassay fluorescent detection was based in a previous development with few modifications (Alonso et al., 2020). Briefly, the equipment was based on the classical setup of a fluorescence microscope, using a proprietary Single-Photon Avalanche Diodes (SPADs) camera for detection. This was enclosed in a 3D-printed case, together with a laser diode, 3 lenses, and 2 filters, giving shape to an economical, compact, and high-performing equipment that was controlled by a Single Board Computer (SBC) with Internet of Things (IoT) capabilities.

2.6 Data analysis

Each paper device was used just once. The calibrates show the average of no less than 3 independent replicates and the error bars correspond to their standard deviation (SD). For each method, blood samples were analysed twice independently.

The limits of detection (LOD) and quantification (LOQ) were calculated as the average of the blanks plus 3 and 10 times their SD, respectively. The variability was calculated in terms of coefficient of variation (% CV = (SD/mean) × 100). The signal-to-noise ratio (S/N) corresponded to the signal registered for each Pf-LDH concentration divided by the signal from the blanks.

3 Results and discussion

Most malaria RDTs are chromatographic-based lateral flow devices that produce qualitative yes/no results in 15–30 min, with little user intervention, and at a cost of 1-5 €/test (Abrahamson, 2018). The objective of this work was to develop a fast test, which could be simpler to produce but as easy to use asclassical RDTs, and at the same time granted quantitative and sensitive detection to facilitate patient stratification and identification of submicroscopic malarias. This goal was achieved by optimising a single-step fluorescent magneto-immunoassay for detection of Pf-LDH; by designing a single-piece paper device to automate most assay steps; and by implementing a portable fluorescence reader that provided fast and sensitive results. The system was finally used to study a battery of clinical samples.

3.1 Optimization of the single-step magneto-immunoassay

Conventional magneto-immunoassays require several incubation and washing steps (Table S-1), which are carried in tubes and using magnets. This level of manipulation requires user training and hampers on-field implementation. In contrast, the single-step magneto-immunoassay optimized here consisted of a single 5-min incubation of the samples with a cocktail of three reagents: c-MAb-MP, bd-MAb and Poly-HRP (Fig. 2a). If Pf-LDH was present in the sample, a sandwich c-MAb-MP/Pf-LDH/bd-MAb/Poly-HRP was formed. MPs were then washed and incubated with an enzymatic substrate (TMB or QuantaRed), which reacted with Poly-HRP to form a coloured or a fluorescent product (oxidised TMB and resorufin, respectively). Three parameters were optimized for this assay to work, which were the amount of c-MAb-MPs, bd-MAb and Poly-HRP needed per sample.

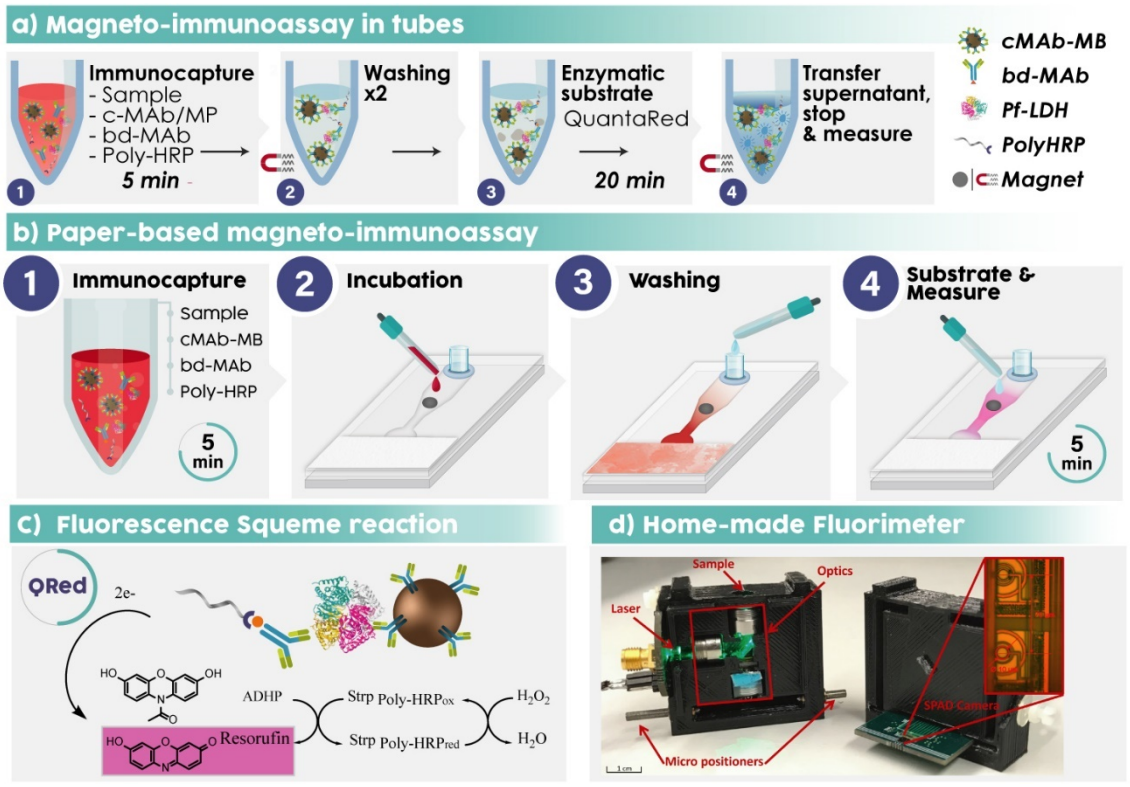

Figure 2. Schematic representation of the single-step magneto-immunoassay operated manually (a) and the partial paper-based automation achieved in this work (b), in which MPs washing, concentration and fluorescent detection are performed directly on-chip. (c) Reaction catalysed by Poly-HRP on QuantaRed. (d) Portable fluorimeter used for detection (Alonso et al., 2020).

The amount of c-MAb-MPs required was determined by detecting increasing concentrations of Pf-LDH with the single-step magneto-immunoassay, using in parallel 10-20 µg of MP per sample. As it can be observed, the higher the concentration of c-

MAb-MPs, the higher the signal and S/N recorded (Fig. 3a-b). A concentration of 20 µg of c-MAb-MPs was thus selected. In the same way, signal increased with the concentration of bd-MAb in the range from 37.5 ng·mL^{-1} to 300 ng·mL^{-1}, but both in the positive and negative controls (Fig. 3c-d). Accordingly, the S/N did not improve for bd-MAb concentrations above 75 ng·mL^{-1}. Finally, Poly-HRP, a commercial conjugate of streptavidin and HRP polymers, was tested in the range between 25 ng·mL^{-1} and 200 ng·mL^{-1}. Again, signals augmented as Poly-HRP concentration raised (Fig. 3e-f). However, the background noise registered in the negative controls (without Pf-LDH) also increased, which was attributed to Poly-HRP nonspecific adsorption. As a result, the highest S/N ratio was observed for 50 ng·mL^{-1} of Poly-HRP.

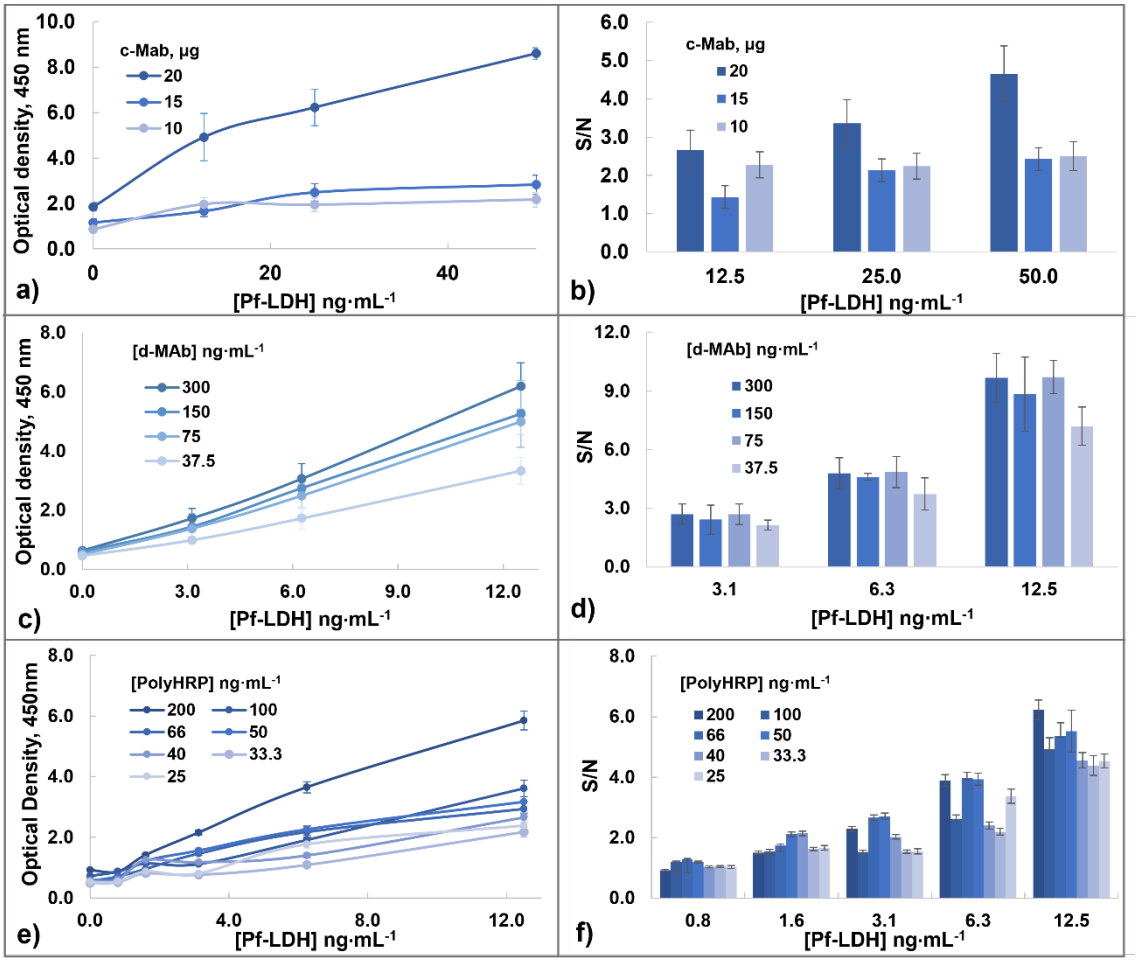

Figure 3. Optimization of amount of c-MAb-MPs, bd-MAb and Poly-HRP per sample in the single-step magneto-immunoassay in tubes. a,c,d) Absorbance and (b,d,f) S/N registered for serial dilutions of Pf-LDH for the variable under study (c-MAb in a-b, bd-MAb in c-d and Poly-HRP in e-f). With each optimization, reproducibility between replicates improved, allowing detection of lower Pf-LDH concentrations.

Upon optimization, the single-step magneto-immunoassay performed in tubes took <30 min, including the immuno-capture, the washing steps and a 20-min incubation with TMB. The linear range spanned between 0.8 ng·mL^{-1} and 25 ng·mL^{-1}, with an LOD of 0.7 ng·mL^{-1} and an LOQ of 1.9 ng·mL^{-1}. TMB was next substituted by QuantaRed, which was incubated for just 5 min before detecting fluorescence. This reduced the total assay time to 15 min. Fluorescence granted a wider linear range (0.2-50 ng·mL^{-1}) and a significant improvement in terms of LOD/LOQ (0.06 ng·mL^{-1} and 0.3 ng·mL^{-1}, respectively; Fig. S-4).

3.2 Detection of Pf-LDH using a paper-based sensor

Membrane selection

A paper device was next designed for the partial automation of the fluorescent magneto-immunoassay, using for detection a miniature fluorescence reader (Canals et al., 2019). The paper device was conceived to carry MP washing under flow conditions and assay end-point fluorescent detection (Fig. 1). An absorbent pad was attached for flow pumping and waste storage. Nine types of membranes were evaluated to produce these devices (Table S-2). Properties such as autofluorescence, solution wicking rate, thickness and protein retention were important to select the material of the paper sensor, while the water absorption capacity was crucial for the adsorbent pad. In the case of the sensor, Stand17, Fusion5 and MF1 were among the membranes that displayed the lowest auto-fluorescence when measured with the reader (544 nm for excitation and 590 nm for emission, the same conditions employed to detect QuantaRed). They exhibited also narrow thickness and low water absorption capacity, which allowed working with small reagent volumes and perform measurements both in the front and back of the sensor (Fig. S-5). Of them, Standard 17, a glass fibre membrane (370 µm thickness) recommended by the supplier for use as a conjugate release pad in lateral flow assays, fulfilled all the requirements exposed above, displaying low protein retention and a fast and homogeneous flowing rate as well (Fig. S-5). The S/N values obtained for Standard 17 when measuring QuantaRed in the presence of HRP were similar in both the front and back of the membrane and were higher than those achieved using Fusion5 and MF1.

On the other hand, to select the best membrane for the absorbent pad, 100 µL of methylene blue were pipetted on one edge of 4×1 cm membrane strips and the distance run by the solution was measured. CF5, GF/DVA and VF2 provided the highest absorption capacity, which was consistent with the data facilitated by the provider (99.2,

93 and 86.2 mg cm^{-2}, respectively; Fig. S-5). Of them, CF5 imparted more homogeneous flow and was selected to produce the device absorption pad.

Design of the paper-based sensor

The standard-17 based sensor consisted of a wide edge, which was the MP washing pad with a volume absorption capacity of 125 µL, a concentration zone where a magnet retained the MPs, a detection channel (or lecture zone), and a protruding end to connect the external absorption pad (Fig. 1a-b). For its utilization, this single-use chip was assembled on a reusable magnetic holder (see section 2.4), which displayed a magnet and a plastic guide for the alignment of the paper sensor.

Three different designs were explored for the washing pad (Fig. 4). The first one, c1, had a rectangular shape with smooth edges and was separated from the MP concentration zone by a narrow neck. The second one, c2, displayed a long and narrow pad that served for washing at one end and for PM concentration at the other. Finally, in c3, the washing pad finished in a funnelled end that served for MP concentration. A plastic reservoir, pasted with Blu-Tack before the washing pad, facilitated solution addition. To carry the paper-based magneto-immunoassay, the 100 µL of sample/cMAb-MP/bd-MAb/Poly-HRP mixture were dispensed at the beginning of the washing pad immediately after the single 5-min incubation (Fig. 2b). Washing buffer was then added to the reservoir. Two additions of 500 µL each were enough to push the MPs towards the MP concentration zone and wash them under flow conditions (Fig S-6). The MPs were retained by the magnet at the MP concentration zone while the excess of reagents and sample were washed away and towards the absorption pad. The enzymatic substrate was finally added to the end of the washing pad to carry detection (Fig. 4 and Fig. S-7).

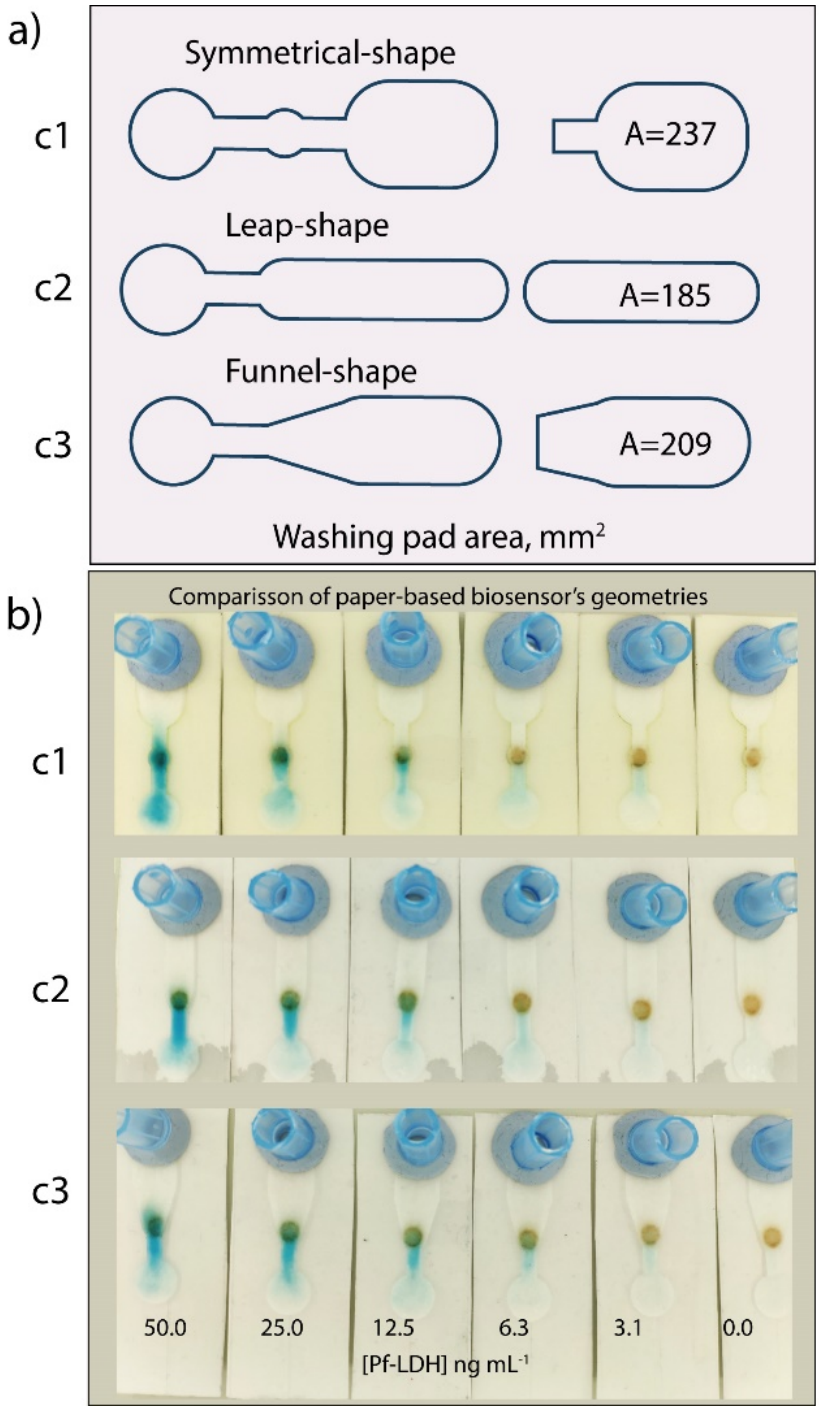

Figure 4. Optimization of the paper-based sensor. a) Geometry of three types of sensors displaying washing pads of different size and shape. b) Detection of a Pf-LDH dilution series using the paper-based single-step magneto-immunoassay employing alternatively the three types of paper-based sensor shown in "a". In all cases, Pf-LHD was incubated in tubes for 5 min with c-MAb-MPs (20 µg), bd-MAb (75 ng mL^{-1}) and Poly-HRP (50 ng mL^{-1}), the mixture was transferred to the distal end of the sensor washing pad and washing solution was added to the reservoir. For detection, 50 µL of substrate solution (TMB) were added to the bottom of the washing pad.

Due to the big washing pad area of c1 (237 mm^2), in this sensor the MPs scattered around the point where they were dispensed, producing variability between replicates

and background signals higher than expected. The washing pad of c2 was too narrow to accommodate all the washing buffer, which had to be pipetted slowly to prevent solution overflow. The best results were obtained with the c3 sensor, which displayed higher and more reproducible signals for all Pf-LDH concentrations and equal or lower background noise than the other two sensors (Fig 4). This was attributed to a better flow of the MPs towards the concentration zone and more efficient washing of leftover reagents away from it. Under these conditions, Pf-LDH was detected in a concentration range spanning between 3.1 and 50 $ng \cdot mL^{-1}$.

Pf-LDH fluorescent detection at the paper sensor

TMB was finally substituted by QuantaRed and the signals generated were measured with the portable fluorimeter. For this, the reusable magnetic holder with the paper-based sensor was placed in a 24x60 mm platform in the fluorescence reader. A series of lenses and mirrors provided alignment of the excitation light beam with the sensor's lecture zone and allowed obtaining the emission fluorescence count in the front of the paper-based sensor with high sensitivity and reproducibility.

Figure 5a displays some examples of the colorimetric signals obtained for increasing concentrations of Pf-LDH using QuantaRed and Fig. 5b shows the calibration plot when the devices were measured with the fluorimeter. Pf-LDH could be detected down to 1.56 $ng \cdot mL^{-1}$ by the naked eye. When measured with the fluorimeter, the lowest concentration of Pf-LDH measured consistently was 0.78 $ng \cdot mL^{-1}$, but the theoretical LOD was of 0.9 $ng \cdot mL^{-1}$ due to the relatively high noise variability. Nevertheless, fluorescence detection using the portable reader provided quantitative detection and lower LOD than colorimetric detection using TMB.

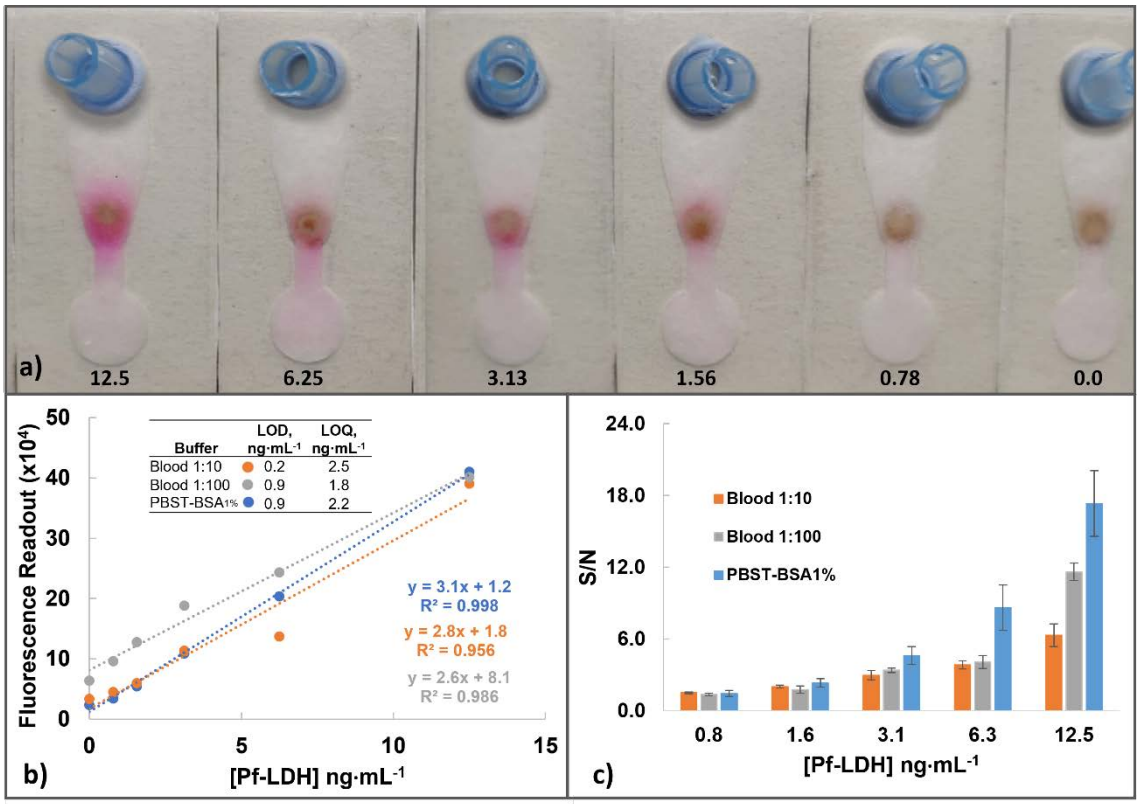

Figure 5. a) Signals generated in the paper-based magneto-immunosensor by increasing concentrations of Pf-LDH (in PBS-BSA$_{1\%}$) using QuantaRed enzymatic substrate. b-c) Intensity and S/N registered with the portable fluorimeter for Pf-LDH (0.78 – 12.5 ng·mL^{-1}) spiked in either PBST-BSA$_{1\%}$ or lysed whole blood (diluted 1:10 and 1:100 with PBST-BSA$_{1\%}$). In all cases, the Pf-LHD-containing sample was incubated in tubes for 5 min with c-MAb-MPs, bd-MAb and Poly-HRP (20 µg, 75 ng mL^{-1}, 50 ng mL^{-1}), the mixture was transferred to the sensor washing pad and washing solution was added to the reservoir. For detection, 50 µL of QuantaRed were added and devices were incubated for 5 min in the dark before taking a picture or measuring fluorescence with the portable reader (λ_{exc} 544 nm, λ_{em} 590 nm).

The fluorescent paper-based magneto-immunosensor was then employed to detect Pf-LDH spiked in lysed whole blood (diluted 1:10 and 1:100). All the calibration plots presented a high correlation between signal and Pf-LDH concentration (R^2 >0.99), and linear ranges spanning between 0.78 ng·mL^{-1} and 12.5 ng·mL^{-1} (Fig. 5b). However, the presence of blood increased the background noise, affecting negatively the S/N and the sensor LOD/LOQ. This was presumably caused by sample components that attached to the paper and were not removed efficiently enough by on-chip washing. This suggested that the study of real clinical samples should be done by interpolation in a calibration curve obtained in a similar blood dilution to avoid result overestimation.

The paper-based fluorescent magneto-immunosensor entailed a total assay time of <15 min, plus 5 min for blood lysis, and displayed linear range (0.78-12.5 ng·mL^{-1}) and LOD (0.90 ng·mL^{-1}) comparable to the colorimetric assay carried in tubes. Furthermore, this LOD was equivalent to approximately 4.5 p·µL^{-1}, which is below the threshold value

recommended by the WHO for classical and ultrasensitive RDTs (200 and 20 p·μL^{-1}, respectively). It has been suggested that POC RDTs capable to detect samples with parasitaemia lower than 20 p·μL^{-1} recognize 95% of malaria-infected samples (The malERA Consultative Group on Diagnoses, 2011). These findings make this POC device a promising technology for malaria diagnosis in resource-limited areas.

3.3 Detection of Pf-LDH in clinical samples from patients

The performance of the paper-based sensor was evaluated further by studying 9 blood samples collected from patients who had *P. falciparum* malaria infection. These samples displayed Pf-LDH concentrations ranging 30-2132 ng·mL^{-1} when studied by ELISA, seven of them were positive by microscopy with parasitaemias spanning 0.1-0.8 %, and the other two were confirmed by PCR and were considered submicroscopic malarias (Table S-3). For their study, samples were treated with lysis buffer for 5 min to release intracellular parasites, were diluted 1:10 with PBS-BSA$_{1\%}$ (except for P9 that had to be diluted 1:100 to be quantified), and were analyzed with the paper-based magneto-immunosensor as described before. Table S-3 and Fig. S-8 compare the results provided for these samples by the sensor, light-microscopy, ELISA, and a commercial RDT.

The paper-based magneto-immunosensor detected Pf-LDH in the 9 samples at concentrations that correlated those obtained by the reference ELISA (30-1530 ng·mL^{-1} in pre-diluted samples, corresponding to 3-153 ng·mL^{-1} in the 1:10 sample dilutions tested in the sensor; Fig. 6a). However, while the sensor provided Pf-LDH quantification in about 20 min, including 5 min of sample pre-treatment, ELISA took nearly 3 h. The sensor was more sensitive than microscopy, detecting two submicroscopic malarias. The correlation between parasitemia and Pf-LDH concentration was limited, and issue that has been noticed by other teams. Here, this was attributed to the fact that microscopy was carried on fresh blood samples, but ELISA and magneto-immunosensing was achieved after sample storage at -80°C. In addition, the POC device exhibited remarkable results when tested against a commercial RDT that provided multiplexed detection of pLDH and Pf-HRP2 (Table S-3). This RDT was positive in 8 out of 9 samples when detecting Pf-HRP2, but only in 4 when detecting pLDH. This was in agreement with the general concern that while most commercial RDT detect Pf-HRP2 with LODs in the range of 0.4–1.6 ng mL^{-1}, the few devices that detect pLDH exhibit LODs spanning 10–1000 ng mL^{-1} (Jimenez et al., 2017). Here, the paper-based magneto-immunosensor afforded malaria identification with higher sensitivity than both Pf-HRP2 and pLDH-based RDT detection. Although a higher number of samples should be studied, these results suggest that the paper-based sensor could be useful to detect malaria in clinical

samples, with results comparable to those provided by current reference methods and a production cost below 0.6 €/test (Table S-4).

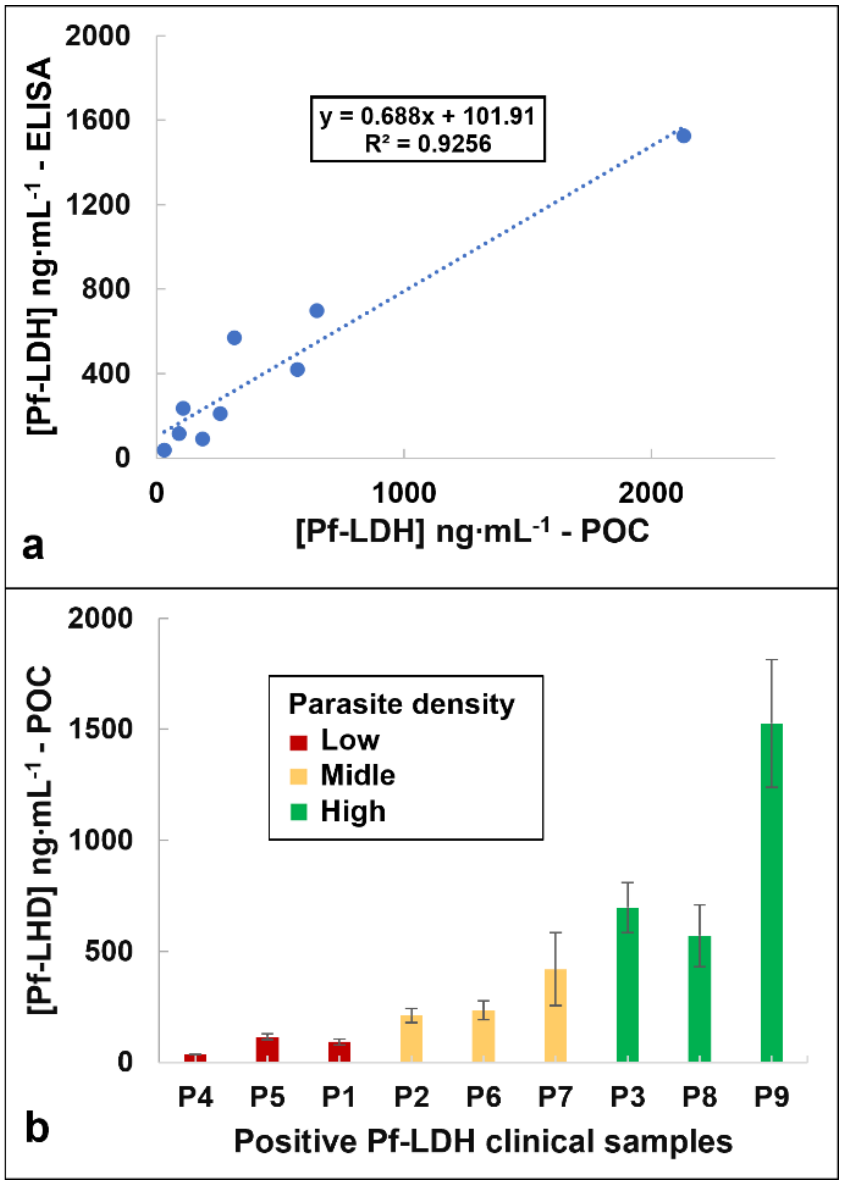

Figure 6. a) Correlation between the concentration of Pf-LDH detected in 9 clinical samples using the paper-based magneto-immunosensor and the reference ELISA. The graphs show Pf-LDH concentration in pre-diluted samples, calculated by interpolating the signals in a calibration plot obtained in spiked blood (1:10; Fig. 5b) and multiplying per sample dilution. b) Patient stratification according to Pf-LDH concentration.

4 Conclusions

In this work, we report a versatile POC device for malaria quantitative diagnosis. Detection of Pf-LDH is based in a single-step magneto-immunoassay, which is partly conducted on a simple and inexpensive paper-based disposable device. Result

interpretation is then carried with QuantaRed, either colorimetrically by the naked eye, or fluorescently using a low-cost fluorescent detector. In <15 min, plus 5-min for whole blood lysis, the system achieves an LOD of 1.56 and 0.9 ng·mL^{-1} for colorimetric and fluorescent detection, respectively. These numbers are equivalent to 7.8 and 4.5 p·µL^{-1}, which are below the cut-off of 20 p·µL^{-1} recommended by the WHO for ultrasensitive RDTs. Furthermore, Pf-LDH detection in clinical samples is more sensitive than microscopy and a commercial RDT, and comparable to the reference ELISA but with less user intervention.

Although a higher number of samples should be analyzed in future, these results suggest that the proposed method could be suitable as a tool for initial diagnosis and massive screening of malaria in the community, which none of the available reference methods affords on its own. Compared to classical RDTs, the new format should be easier to tune, for example to accomplish *Plasmodium* species-specific detection or identification or alternative microorganisms, by changing the c-MAb-MP and bd-MAb. Future improvement to achieve complete automation of sample pretreatment and MP manipulation should take this development a step closer to a commercial prototype.

Acknowledgements

This work was supported by *Instituto de Salud Carlos III (ISCIII)* and the European Regional Development Fund (grants CPII18/00025, IFI18/00020 and QUPID EuroNanoMed AC21_2/00021). KAA is supported by an InPHINIT fellowship from ”La Caixa” Foundation (LCF/BQ/DI18/11660061). ASM is supported by a *Juan Rodés* postdoctoral fellowship from ISCIII *(JR18/00022)*. Diagnostic Nanotools is an AGAUR Consolidated Group (2017 SGR 240).

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Malaria quantitative POC testing using magnetic particles, a paper microfluidic device and a hand-held fluorescence reader

Supplementary Information

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Material and Methods

Production of biotinylated detection antibodies (bd-MAb).

Briefly, a buffer exchange was performed to remove interfering reagents from the d-MAb. First, 300 μg of d-MAb were placed in an Amicon® Ultra 0.5 mL centrifugal filter (Merk Millipore). The volume was adjusted to 0.5 mL with carbonate-bicarbonate buffer (0.1 M, pH 9.5). Then, the tube was centrifuged at 14000g for 10 min, the filter was filled again with 0.5 mL of carbonate buffer and was centrifuged once more. The concentrated d-MAb was then recovered, and carbonate-bicarbonate buffer was added to bring the antibody to a final volume of 0.2 mL.

A fresh stock of biotin-XX (6-((6-((Biotinoyl)Amino)Hexanoyl)amino)Hexanoic Acid, Sulfo-succinimidyl Ester, Sodium Salt; Ref. B6352, Thermo Fisher Scientific) was prepared at a concentration of 2.5 $mg \cdot mL^{-1}$ in MILI-Q water and 9.66 μL were added to the d-MAb. The mixture was stirred at 24°C in the dark for 2 h. A G25 desalting column (GE Healthcare) was used to separate the bd-MAb from unbound biotin, eluting 0.2-mL fractions using PBS. The concentration of the bd-MAb was checked by UV-Vis spectroscopy, it was adjusted to 150 $\mu g \cdot mL^{-1}$ with PBS, BSA 1%, and the bd-MAb was finally stored in working aliquots at -20 °C.

Figure S-1. MP functionalization with c-MAb.

MPs (1 mg in 100 µL) were washed twice with 15 mM MES using a magnetic separator (BILATEST, Sigma Aldrich). MPs were then mixed for 15 min in 100 µL of a solution of 2 $mg \cdot mL^{-1}$ of EDC and 0.25 $mg \cdot mL^{-1}$ of c-MAb (950 rpm, thermoshaker Thermal Shake lite, Avantor, USA). After that, MPs were serially washed with 200 µL of MES and with 200 µL of PBS, and were blocked for 1 h with PBS-$BSA_{1\%}$. The c-MAb-MB were then washed for 5 min with 100 µL of PBS, Tween 20 0.1% (PBST) and were resuspended in 500 µL of PBS, Tween 20 0.1%, BSA 0.2% (PBST-$BSA_{0.2\%}$) for storage at 4°C (final concentration of 1.4-2.4×10^9 MPs·mL^{-1}, equivalent to 2 $mg \cdot mL^{-1}$). Under these storage conditions, c-MAb-MB were stable for at least 1 month (longer storage times have not been tested). The following figure illustrates the chemical reaction involved.

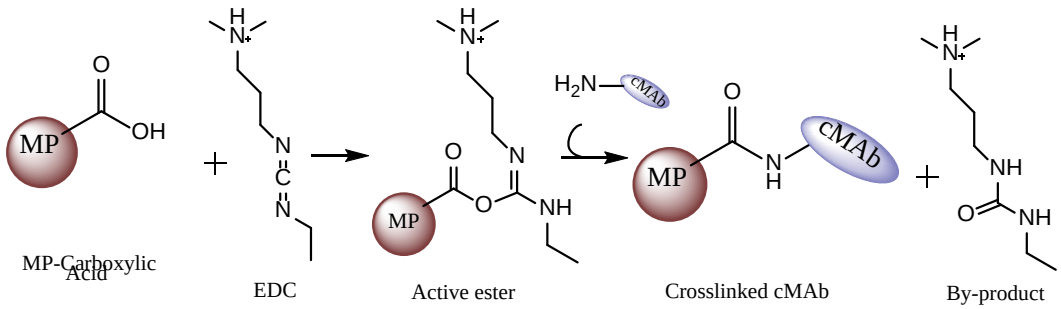


Figure S-2. Scheme and protocol of the reference sandwich ELISA for Pf-LDH detection.

An ultrasensitive shortened ELISA reported previously was employed as reference methodology for the study of the lysed whole blood samples (de la Serna et al., 2021). Briefly, microtiter plates were modified for 1 h with c-MAb (2.5 µg mL^{-1} in PBS) and were blocked with PBST-$BSA_{1\%}$ for 1 h, followed by a 30-min incubation with bd-MAb (37.5 ng mL^{-1} in PBST-$BSA_{1\%}$). The plate was then washed, incubated for 10 min with polyHRP (1:10000 in PBST-$BSA_{1\%}$). After four more washes, the plate was incubated for 20 min with TMB before stopping the reaction with sulphuric acid (50 µL $well^{-1}$) and measuring the absorbance at 450 nm using a Sunrise plate reader (Tecan, Switzerland).

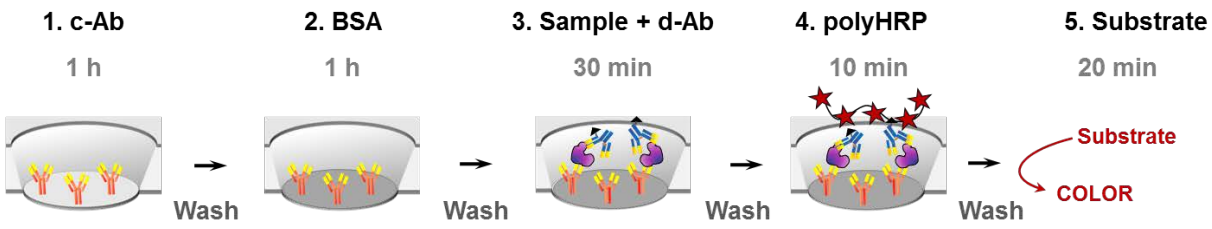


Figure S-3. Two-step magneto-immunoassay for Pf-LDH detection.

The 2-step magneto-immunoassay reported in (Sánchez-Cano et al., 2021) started with c-MAb-MP washing twice with PBS, followed by c-MAb-MP resuspension in 1×RD to 5 mg mL^{-1}. Unless otherwise specified, all subsequent incubations were performed in 100 µL of 1xRD, in Eppendorf tubes, under agitation at room temperature, and were followed by 2 washes with 150 µL of PBS supplemented with 0.05% of Tween 20 ($PBST_{0.05\%}$).

For the assay, Pf-LDH-containing samples were mixed with 25 µg of c-MAb-MP and 75 ng of bd-MAb and the mixture was stirred for 5 min at 950 rpm. After washing, MP were incubated for 5 more min with Poly-HRP (50 ng mL^{-1}) and were washed twice with $PBST_{0.1\%}$ and once with PBS. MP were then incubated with 100 µL of QuantaRed fluorescence substrate solution in a thermoshaker at 1500 rpm for 15 min. The supernatant was transferred to a black polystyrene 96-well plate (Corning), where the reaction was stopped and measured at 544 nm for excitation and 590 nm for emission using a FLUOstar Omega plate reader (BMG Labtech, Germany).

This 2-step magneto-immunoassay displayed linear response between 0.1 and 25 ng mL^{-1} of Pf-LHD, with LOD/LOQ of 0.11 and 0.20 ng mL^{-1}, respectively.

Note: QuantaRed is a commercial kit that includes three solutions that have to be mixed immediately before their use (QuantaRed ADHP concentrate, QuantaRed enhancer solution, and peroxide solution), and a stop solution to terminate the enzymatic reaction after a 15-min incubation.

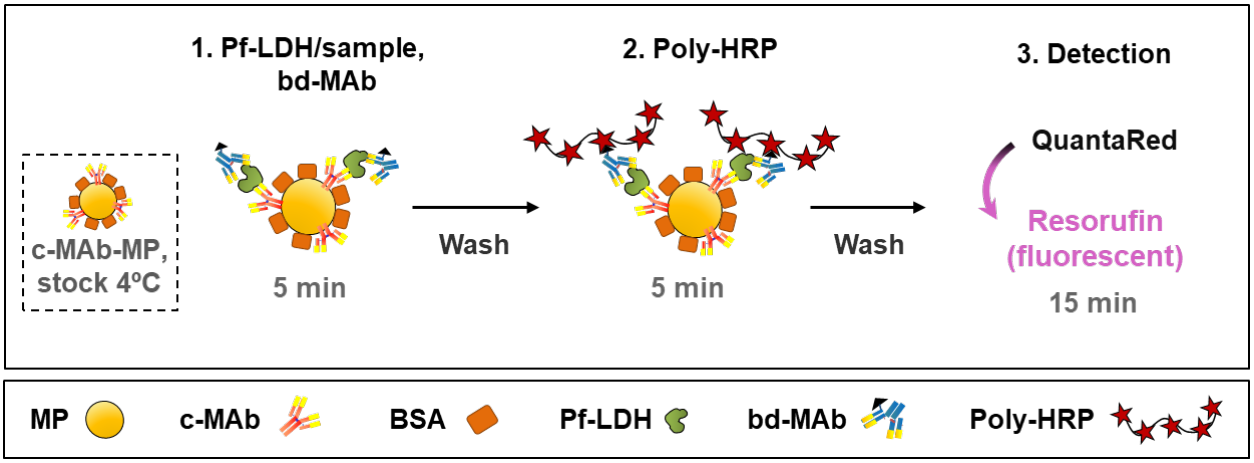


Figure S-4. Single-step magneto-immunoassay for Pf-LDH detection: comparison of colorimetric and fluorescent detection. The 2-step magneto-immunoassay was then replaced for a shorter, simpler, and faster single-step immunoassay version. This magneto-immunoassay consisted in a single 5-min incubation of the sample (diluted with 1×RD to a final volume of 95 µL) with c-MAb-MPs (4 µL), bd-MAb (75 ng·mL^{-1}) and Poly-HRP (50 ng·mL^{-1}). MPs were then washed twice with 150 µL of PBST, and were stirred for 20 min in 100 µL of enzymatic substrate solution (TMB or QuantaRed). After this, MPs were concentrated and the supernatant was transferred to 96-well plates. For colorimetric detection, 50 µL of 1 M sulphuric acid were added to each well and absorbance was measured at 450 nm using a Sunrise plate reader (Tecan, Switzerland). For fluorescence detection, the supernatant was transferred to a black 96-well plate, 10 µL of stop solution were added and fluorescence was measured using a FLUOstar® Omega plate reader (BMG Labtech, Germany; 544 nm for excitation and 590 nm for emission).

The single-step magneto-immunoassay was developed using an inexpensive and ready-to-use chromogenic enzymatic substrate solution, TMB. Despite the good results obtained in the colorimetric magneto-immunoassay performed in tubes (LOD=0.7 ng·mL^{-1}, LOQ=1.9 ngmL^{-1}, linear range= 0.8 - 25 ng·mL^{-1}), the assay had a total assay time of nearly 30 min. To improve the sensitivity and decrease the total assay time, TMB was substituted by a fluorescent enzymatic substrate (QuantaRed). This change reduced the total assay time in tubes down to 15 min. Fluorescence granted a wider linear range (0.2 - 50 ng·mL^{-1} and a significant improvement in terms of LOD/LOQ (0.06 ng·mL^{-1} and 0.3 ng·mL^{-1}, respectively).

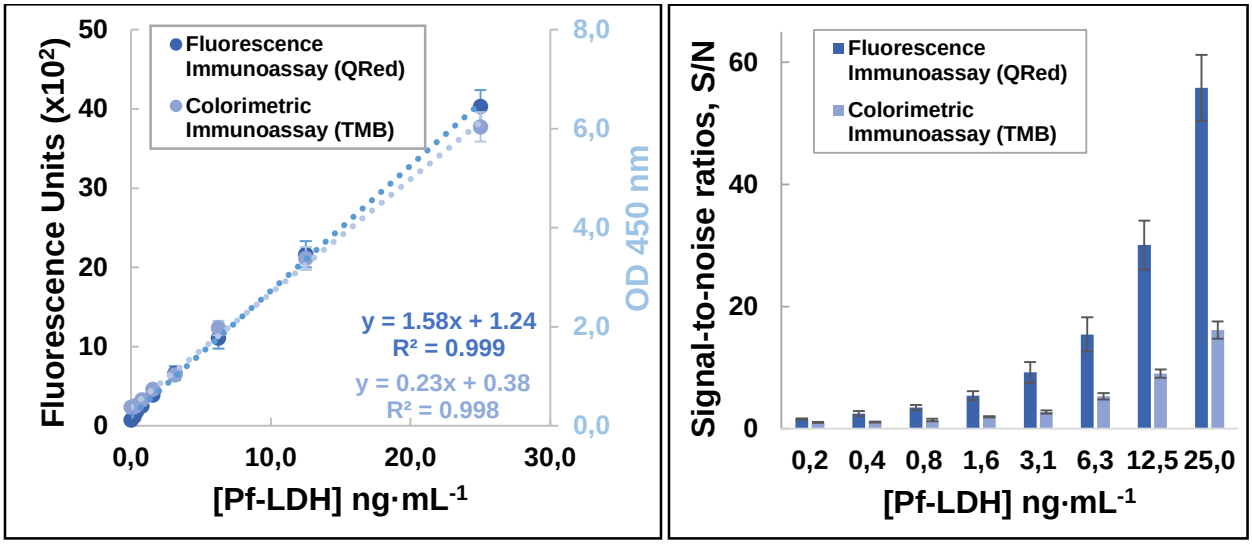
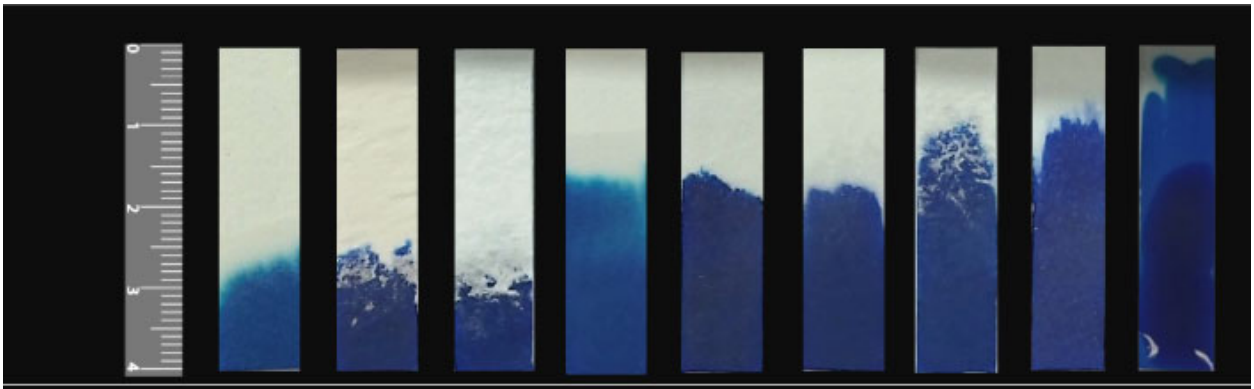


Figure S-5. Performance of the 9 membranes studied.

(Top) Study of the absorption capacity of the 9 paper-like membranes. Methylene blue (100 µL) was pipetted in one edge of a 4×1 cm membrane strip to measure the distance run by the solution. (Bottom) Membrane main properties. Thickness, wicking rate, and water absorption capacity (Water abs) were obtained from the supplier. Auto-fluorescence and signal-to-noise ratios (S/N) were measured using the hand-held reader before and after adding QuantaRed substrate solution. For this, QuantaRed was incubated with HRP and was pipetted in the front of the strip. Fluorescence was then measure both in the front and the back of the membrane strip.



Material	CF5	GF/DVA	VF2	CF4	Stand17	Fusion5	MF1	LF1	Prima40
Autofluorescence	High	Low	Medium	High	Low	Medium	Low	Medium	Medium
Thickness (µm)	954	785	785	482	370	370	367	247	192
Wicking rate (s·4cm)	66.3	28.2	23.2	67.3	34.5	38	29.7	35.6	44
Water abs (mg·cm^{-2})	99.2	93	86.2	49.9	44.9	40	39.4	25.3	--
Maximal S/N - Front	2.2	13.5	19	45	340	140	170	190	21
Maximal S/N - Back	42	460	440	74	360	340	560	250	50

Figure S-6. Optimization of MP washing on-chip.

Replicates of the single-step magneto-immunoassay performed in the paper-based sensor c1 after different washing procedures:

- 1 wash with 500 µL of PBST;
- 2 washes with 500 µL of PBST each.
- 1 wash with 500 µL + 2 washes with 250 µL of PBST each;

For this experiment, the single 5-min incubation of the magneto-immunoassay was carried in tubes. The 100-µL of sample/cMAb-MP/bd-MAb/polyHRP mixture was then dispensed at the beginning of the washing pad. This was followed by the appropriate washing procedure. The enzymatic substrate, QuantaRed, was finally added to the MP concentration zone and colour evolution was interpreted visually over time. As it can be seen, as the washing volume increases the background signal decreases in the negative controls, while signal reproducibility improves in the positive controls. This shows that unspecific reagent absorption is taking place and causing result variability in the absence of appropriate washing. The best results were obtained when carrying two consecutive additions of 500 µL of PBST, which produced more homogeneous and reproducible patterns in the positive controls and lower background noise in the negative controls.

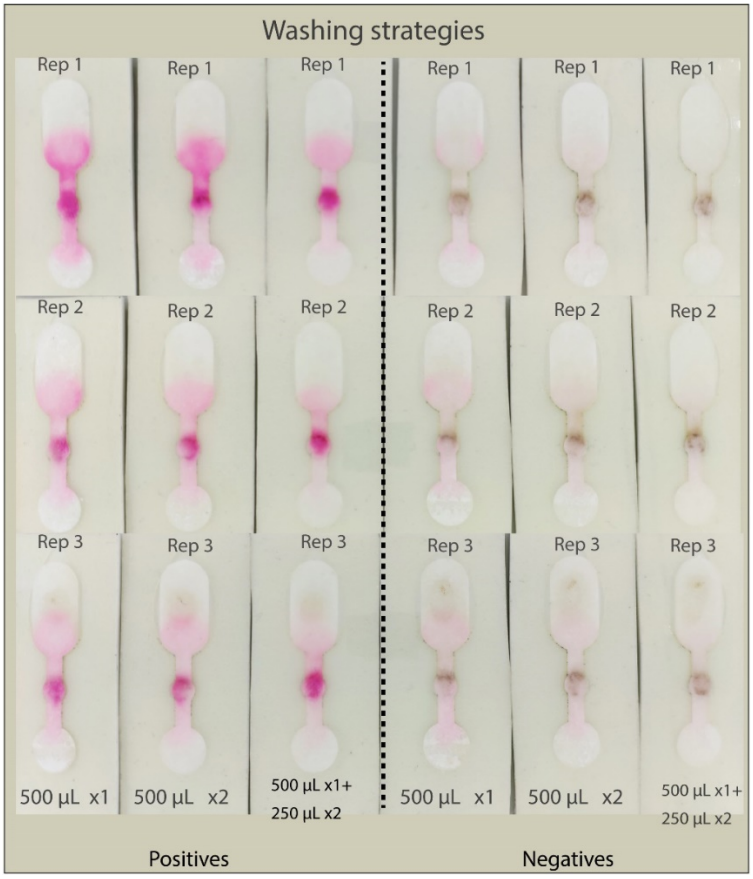


Figure S-7. Optimization of the paper-based detection strategy.

Colorimetric signals obtained in the paper-based magneto-immunoassay for a Pf-LDH serial dilution (3.13 - 50 ng·mL^{-1} of Pf-LDH). For detection, 50 µL of TMB were dispensed 5 mm upstream from the magnet location. The absorbent pump was alternatively removed or left in place while the 5-min TMB incubation was happening in the dark. This experiment was carried in parallel using paper-based sensors c1 (left) and c3 (right). As it can be observed, removing the absorbent pump contributed to the dispersion of the coloured enzymatic product around the MP retention zone (both upstream and downstream). Results displayed in this case presented a lower colour intensity in the positive controls but higher background noise in the negatives, especially in the c1 sensors. On the other hand, if the absorbent pump was not removed, the enzymatic substrate solution flowed towards the bottom of the device. Although the absorbent pump was designed to be almost saturated at this point of the assay, slight but noticeable solution flow still took place, which was presumably driven by solution evaporation. This improved significantly the positive signals while the negative controls were still low. In this case, it can be seen that the funnelled c3 sensor displayed higher colour intensities than its c1 counterpart.

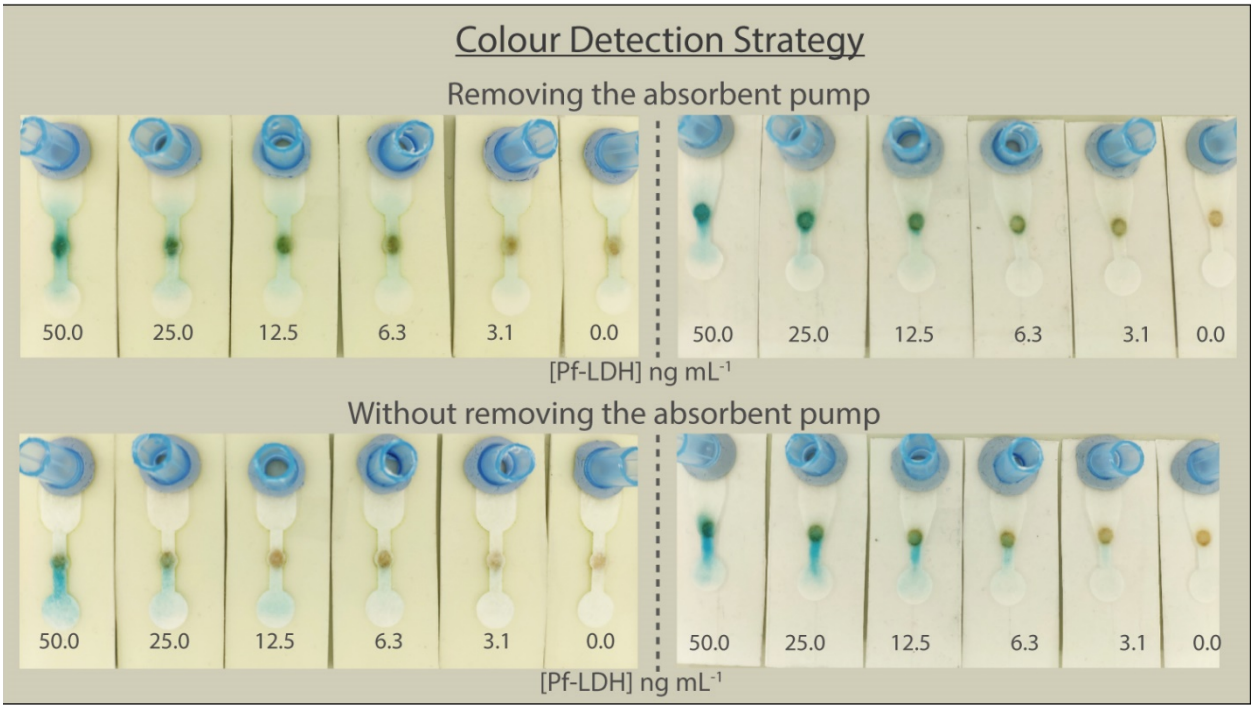


Figure S-8. Detection of malaria in positive blood samples using a commercial RDT.

The nine clinical samples were analysed in parallel using a commercial RDT (SD BIOLINE Malaria Antigen P.f./Pan RDT, ABBOT - formerly Alere - ref. 5FK60), following the instructions provided by the supplier. This device displays a control line and two test lines for multiplexed detection of pan LDH (pLDH) and *Plasmodium falciparum* HRP2 (Pf-HRP2).

Only P5 was clearly negative for both Pf-HRP2 and pLDH.

Four out of p samples were positive or faintly positive for both Pf-HRP2 and pLDH: P1, P3, P7 and P9.

The other 5 samples (P2, P4, P5, P6 and P8) were positive only for Pf-HRP2.

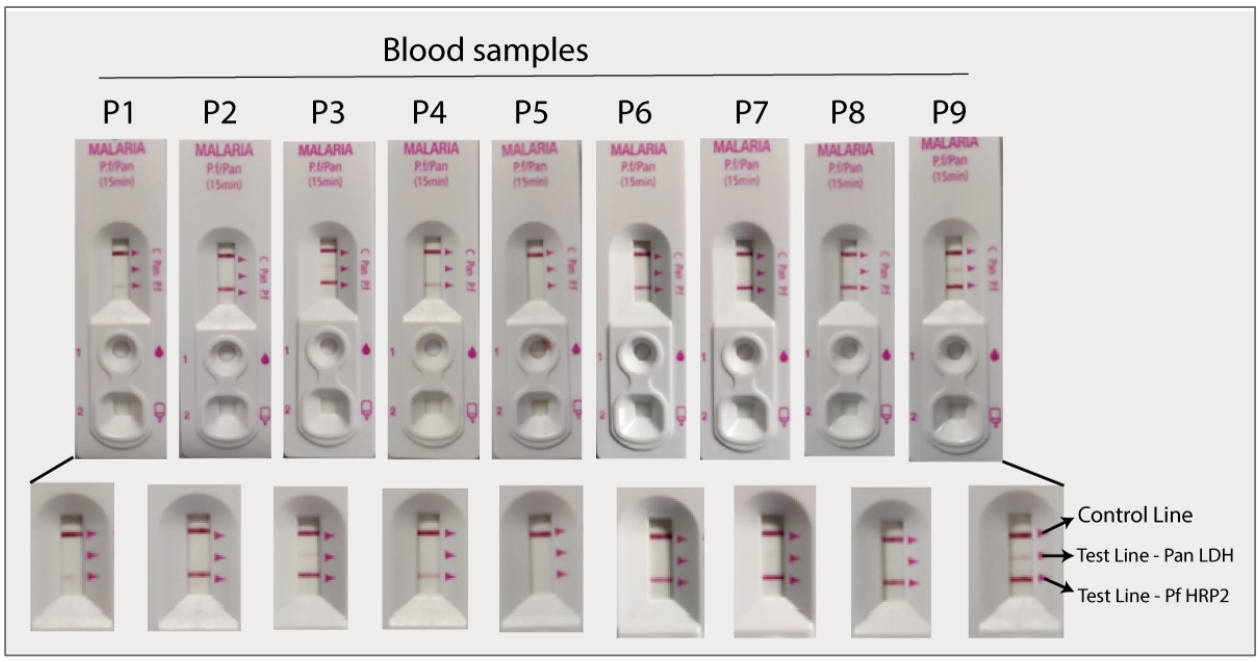


Table S-1. Table S-1. Examples of magneto-immunoassays reported before for malaria diagnosis. The number of assay steps and assay time include the different incubations, but not the washes, which are not detailed in all the works reviewed, or detection, which is stated separately. PLDH, *Plasmodium* lactate dehydrogenase (LDH); PfLDH, *Plasmodium falciparum* LDH; HRPII, histidine rich protein; QD, quantum dots; QR, QuantaRed.

* According to the provider, the recombinant PfLDH protein used in this work has a molecular weight of approximately 65 kDa.

** Does not include the 5 min required for sample pre-treatment (blood lysis).

Assay format	Detection method	Target	MB modif. time	Assay steps	Assay time	Det. time	Linear assay range	LOD	Samples	Ref.
Direct	Colorimetric	PfLDH	1 h	2	45 min	30 min	-	25.7 ± 1.1 pM	*P. falciparum* spiked whole blood	Markwalter 2016a
Direct	Fluorescent	HRP-II PfLDH PvLDH	12 h	6	14.5 h	30 min	0.007-0.763 ng mL^{-1} 0.008–17 ng mL^{-1} 0.008–17 ng mL^{-1}	0.006 ng mL^{-1} 0.056 ng mL^{-1} 1.093 ng mL^{-1}	2031 plasma, serum, EDTA whole blood	Martiáñez 2020
Sandwich	Colorimetric	PLDH PfHRP-II	1 h	2	35 min 25 min	15 min 5 min	7.0-500 pM 1.5-80 pM	2.6 ± 1.5 pM 1.6 ± 1.0 pM	Commercial pooled human whole blood	Markwalter 2016b
Sandwich	Colorimetric	HRP-II	36 h	1	2 h	30 min	1.3 – 62.5 ng mL^{-1}	1.31 ng mL^{-1}	12 patient serum samples	De Souza 2011

Sandwich	Colorimetric	HRP-II	-	4	60 min	30 min	Qualitative	1.0 parasite·µL^{-1}	*P. falciparum* spiked blood	Ricks 2016
Sandwich	Fluorescent (QD)	PfLDH / PvLDH	3 h	3	110 min	5 min	0.51-15 fmols	0.001 – 0.066 ng mL^{-1} (10 – 1000 amols)	-	Kim 2017a
Sandwich	Fluorescent (QD)	HRP-II	1 h	4	90 min	15 min	0.1-10 ng mL^{-1}	Vial (0.1 ng mL^{-1}) Droplet (2.5 ng mL^{-1})	-	Kim 2017b
Sandwich	Colorimetric Fluorescent Chemiluminescent	PfLDH	1 h 25 min	2	10 min	20 min 15 min 1 min	0.4-12.5 ng mL^{-1} 0.1-25 ng mL^{-1} 0.04-12.5 ng mL^{-1}	0.12 ng mL^{-1} 0.11 ng mL^{-1} 0.02 ng mL^{-1}	7 patient lysed whole blood samples	Sánchez-Cano 2021
Sandwich (paper electrode)	Electrochemical	PfLDH	3 h	1	5 min	5 min	6.25-100 ng mL^{-1}	2.51 ng mL^{-1}	3 patient lysed whole blood samples	Ruiz-Vega 2020
Sandwich	**Assay in tubes** Colorimetric (TMB) Fluorescent (QR) **Paper-based assay** Colorimetric (TMB) Colorimetric (QR) Fluorescent (QR)	PfLDH	1 h 25 min	1	5 min**	20 min 5 min 20 min 5 min 5 min	0.8-25 ng mL^{-1} 0.2-50 ng mL^{-1} 3.1-50 ng mL^{-1} 1.56-50 ng mL^{-1} 0.78-12.5 ng mL^{-1}	0.7 ng mL^{-1} (11 pM)* 0.6 ng mL^{-1} (9 pM)* 3.1 ng mL^{-1} (48 pM)* 1.56 ng mL^{-1} (24pM)* 0.9 ng mL^{-1} (14 pM)*	9 patient lysed whole blood samples	This work

Table S-2. Characteristics of the 9 paper-like membranes tested.

Nine types of membranes (GE Healthcare) were evaluated for the production of the paper-based sensor and adsorbent pad.

(-) indicates that this data was not facilitated by the supplier.

Membrane	Material	Properties	Lateral flow application	Thickness (µm)	Wicking rate (s/4cm)	Water absorption (mg cm^{-2})
LF1	Bound glass fibre	For whole blood or serum samples and as a blood separator as well	Blood separation	247	35.6	25.3
MF1			Blood separation	367	29.7	39.4
VF2			Blood separation	785	23.8	86.2
Standard 17		Faster flow than cotton, with lower sample retention	Conjugate release	370	34.5	44.9
GF/DVA		For saliva samples and as a blood separator also	Blood separation	785	28.2	93
CF5	100% cotton linter	Medium weight	Absorption pad	954	63.3	99.2
CF4			Sample application & absorption pad	482	67.3	49.9
Fusion 5	Glass fibre with a plastic binder	Can be used as a lateral flow blood separator	Blood separation & conjugate release	370	38.0	40
Prima 40	Nitrocellulose	Backed membrane to increase mechanical strength	Backed reservoir pad for saliva	192	44.0	_

Table S-3. Summary of results obtained in malaria-positive clinical samples using the paper-based fluorescence POC and the reference ELISA, microscopy and RDT methodologies.

Blood samples from 9 patients infected with *P. falciparum* were analysed by microscopy, ELISA, a commercial RDT (detecting both biomarkers Pf-HRP2 and pLDH), and the paper-based magneto-immunosensor developed in this work. Quantitative results were obtained by measuring fluorescence using a customized fluorimeter (λ_{exc} 544 nm, λ_{em} 590 nm). Two independent replicates were obtained per sample. The concentration of Pf-LDH was calculated by interpolation of the fluorescence counts in a calibration plot obtained for spiked blood (1:10; Fig. 5b in the main manuscript) and multiplying per the dilution factor. Accordingly, concentrations correspond to concentration of Pf-LDH in pre-diluted blood samples.

Sample	ELISA	Fluorescent POC	RDT		Microscopy
	ng mL^{-1}	ng mL^{-1}	Pf-HRP	Pan LDH	Parasitaemia, %
P1	+ (91)	+ (92)	+	+	+ (0.3)
P2	+ (240)	+ (210)	+	-	+ (0.6)
P3	+ (649)	+ (698)	+	+	+ (0.3)
P4	+ (30)	+ (37)	+	-	- (<0.1)
P5	+ (91)	+ (115)	-	-	- (<0.1)
P6	+ (106)	+ (234)	+	-	+ (0.2)
P7	+ (570)	+ (420)	+	+	+ (0.1)
P8	+ (316)	+ (570)	+	-	+ (0.8)
P9	+ (2132)	+ (1527)	+	+	+ (0.8)

Table S-4. Estimated production cost for each paper-based magneto-immunoassay test.

The study includes only reagents and materials.

The estimated cost per test includes the cost of the different components of the magneto-immunoassay, plus the cost of production of the paper-based device, as detailed below. Most of it, 0.410 € (67% of the total), corresponds to the immunoassay reagents, especially PM and QuantaRed. On the other hand, the cost of the disposable paper sensor represents 0.205 €/test (33% of the total), being the absorbent pads the component which contributes the most. All the prices listed here were find in the corresponding supplier catalogue/website, and special discounts potentially available (such as for large purchases) were not taook into consideration.

Reagent	Comercial unit	Price / unit	Amount / test	Tests/unit	Cost / test
PM	Bottle, 100 mg	606.00 €	20 µg	5000	0.1212
c-MAb	Eppendorf, 1 mg	188.00 €	0,5 µg	2000	0.0940
bd-MAb	Eppendorf, 1 mg	188.80 €	8,25 ng	117647	0.0016
Poly-HRP	Eppendorf, 0.25 mg	258.00 €	5.5 ng	45454	0.0057
QuantaRed	3-reagent kit, 110 mL	412.00 €	50 µL	2200	0.1873
Sensors (Standard17)	Pack x 50 A3 sheets	436.00 €	1 sensor	5700 (114/A4)	0.0765
Absorbent pads (CF5)	Pack x 50 A3 sheets	399,46 €	1 pump	3600 (72/A4)	0.1110
			Total cost/ test		**0.5972**

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