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Creating an improved workflow for paper-based malaria diagnostics by integrating total lysis of whole blood

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Detection of parasite biomarkers is critical for diagnosis and treatment monitoring; however, proper analysis of whole blood samples typically requires red blood cell lysis. While lysis is easily achieved in laboratory environments, there is a clinical need for low-cost technology to collect, process, and analyze these samples in low-resource settings. Dried blood spot cards offer a simple way to microsample blood in these settings, but standard cards cannot perform sample preprocessing, including the required lysis step. To meet this need, we have developed a three-dimensional paper-based lysis device that was fabricated alternatively by laser cutting and by wax-printing. This device effectively achieved 100% cellular lysis independent of sample composition. Successful lysis from these devices resulted in the detection of a malaria biomarker comparable to the standard liquid lysis procedure of *Plasmodium*-infected samples. Moreover, if device-dried, samples displayed antigen stability for over 1 week at room temperature, which facilitated delayed laboratory testing as well. The results show that these lysis devices have the potential to expand the types of environments from which samples tested for malaria can be collected.

1. Introduction

Traditionally, diagnostic testing depends on collecting blood *via* venipuncture by trained staff, followed by testing at centralized health care facilities. Though these systems are well established, they are incompatible with point-of-care (POC) testing, which necessitates new methods of blood collection. This need fostered the development of technologies that enable self-collection of blood microsamples under simple and relatively standardized conditions.[1,2] Nowadays, rapid diagnostic tests are available that can be operated with very small amounts of blood, which can be easily obtained from capillary finger-prick sampling.[1] By implementing economical lancets and dried blood spot (DBS) cards, patients can procure their own samples *via* fingerstick.[3] While some testing procedures can be performed directly from these DBS cards, others require additional processing of the blood samples prior to analysis, such as removal of cellular components, cell lysis, or sample dilution. Standard DBS cards cannot incorporate these pre-processing steps, relegating their use to simple collection tools. Accordingly, sample preprocessing has been usually carried off chip by authors reporting rapid methods for malaria diagnosis.[4,5] In this context, paper-based devices can facilitate capillary blood collection with fewer environmental impacts and power requirements than those often seen in more sophisticated strategies.[6,7] Multifunctional devices have been developed from layering different paper-like materials, so that the users can acquire, process, separate, and even store different blood components with minimal handling. For instance, paper-based devices can incorporate a porous membrane to passively separate plasma[8] or paper preloaded with a hemolytic buffer to induce whole blood lysis.[9]

Whole blood lysis is usually required for the analysis of intracellular red blood cell (RBC) components, whether they are endogenous, such as hemoglobin and ferritin, or exogenous, like the antigens or nucleic acids of some parasitic microorganisms.[10,11] Complete RBC lysis results in an increase in the concentration of measurable antigen thereby improving assay analytical performance of these assays. This capability is particularly important when diagnosing diseases caused by parasites (*e.g.*, *Babesia*) and bacteria (*e.g.*, *Bartonella*) that invade

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RBCs,[12] where diagnostic biomarkers are detected at lower concentrations in plasma, serum, or whole blood as compared to lysed blood samples.[13] Perhaps the most notorious of these diseases is malaria, which is caused by *Plasmodium* parasites. *Plasmodium* displays a complex life cycle that includes an intraerythrocytic stage, during which the parasite actively replicates within the host RBCs. This stage corresponds to the period when patients experience malaria symptoms. Accordingly, when malaria is diagnosed in plasma, serum, or untreated whole blood, intraerythrocytic parasites and their associated biomarkers can escape detection. It has been widely recognized that detection of *Plasmodium falciparum* antigens improves when blood is lysed prior to testing, allowing release of parasite biomarkers into the sample.[13] However, blood lysis is a procedure that is regularly carried out as a separate step, independent of the assay itself, often entailing tedious pre-processing steps and biosafety risks for the user.[14] Despite this clear need, commercially available blood microsampling devices do not currently enable the production and storage of whole blood lysates for delayed analysis.

To fill this gap, we developed a three-dimensional paper-based device to passively lyse the RBCs, separate the cellular debris, and store the hemolysate for later analysis. The final device consisted of three layers of porous materials to achieve each of these three steps, including a paper-based lysis component pre-imbedded with a hemolytic reagent (saponin or Triton X-100). This device achieved 100% lysis of RBCs in whole blood with hematocrits (*i.e.*, cellular fraction of whole blood) ranging between 25–55%, and achieved lysis of both capillary and venous blood without anticoagulant dependency. Additionally, the device was employed to collect and lyse clinical samples and RBCs infected with *P. falciparum*, the most deadly and widespread *Plasmodium* species causing human malaria.[15] These samples of stored hemolysate were then processed by ELISA to quantify *P. falciparum* lactate dehydrogenase (Pf-LDH), a standard malaria biomarker. Samples processed using our lysis devices yielded concentrations that were, on average, four times higher than those obtained from unprocessed whole blood and on par with concentrations measured using standard liquid lysis approaches. The results show that these devices have the potential to aid in the expansion of POC technologies for the detection of *Plasmodium* and other intraerythrocytic pathogens.

2. Materials and methods

2.1. Reagents and biocomponents

Recombinant Pf-LDH was provided by Span diagnostics (Venette, France). Monoclonal capture and detection antibodies to Pf-LDH (C01833M and C01835M, cMAb and dMAb, respectively) were from Meridian (Memphis, TE, USA). The latter were biotinylated (bt-dMAb). Streptavidin-conjugated polymerized HRP (polyHRP, Ref. 21140), 1-Step Ultra TMB-ELISA (TMB; Ref. 34029, including TMB and H_2O_2 at undisclosed concentration) and EDTA (Ref. AM9260G) were obtained from Thermo Fisher (Waltham, USA). Bovine serum albumin (BSA), Triton X-100, 1 M imidazole and Tween-20, were obtained from Merck (Madrid, Spain). Phosphate-buffered saline (PBS) was prepared with 10 mM sodium phosphate, 140 mM NaCl, 2.7 mM KCl at pH 7.4. Reagent diluent (10× RD, Ref. DY995; equivalent to 10× PBS, 10% BSA) was obtained from R&D (Abingdon, UK). Saponin (Ref. 558255-100GM) was obtained from Millipore (Burlington, MA, USA). Drabkin's reagent and Brij 35 (30% w/w) were obtained from Ricca Chemical (Arlington, TX, USA). Lyophilized hemoglobin standard was obtained from Pointe Scientific (Canton, MI, USA). 40 mm microhematocrit capillary tubes were purchased from LW Scientific (Lawrenceville, GA, USA) and Ficoll-Paque PLUS was obtained from Cytiva (Marlborough, MA, USA). In this work, PBST indicates PBS with 0.05% Tween 20; PBS–BSA indicates PBS with 1% BSA, prepared by diluting 10× RD; and PBST–BSA combines both.

2.2. Paper-based blood lysis device

The three-dimensional paper-based lysis device comprises a series of laminated layers of porous materials, double-sided adhesive and laminate. The different layers were designed using Adobe Illustrator to provide four functions (Fig. 1). The inlet at the top, an orifice 5 mm in diameter cut in the laminate cover, facilitated sample addition, where 60 μL of whole blood was applied. In the next layer, blood reconstituted a stored reagent that resulted in total hemolysis. To remove any unwanted cellular debris from the final sample, a plasma separation membrane was positioned underneath the lysis layer so the hemolysate could flow uninterrupted to the sample outlet. On the bottom, a patterned paper channel guided the lysed blood towards the outlet for its recovery. The lysed blood could then be either stored *in situ* or recovered using a punch for immediate elution and testing. In both cases, hemolyzed samples should fill the outlet completely before sample recovery.

We explored two different versions of the device, each manufactured with different patterning methods. In the first version (**Version 1**), the fluidic paths were defined *via* wax-patterning, using saponin as the lysing agent, based on a previous design of a paper-based lysis device (Fig. 1, left).[9] These patterns were wax-printed using a Xerox ColorQube 8580 wax printer (NY, USA) first onto Avery laminate sheets (purchased from Amazon) before transferring onto the paper layers (Munktell TFN, Laboratory Sales & Service LLC, Somerville, NJ, USA) using a double-sided printing method.[16] The wax was then melted into the paper with a Promo Heat CS-15 T-shirt press for 45 seconds at 140 °C to achieve total wax penetration and defined hydrophobic barriers. Between lysis and channel components, a disk of a plasma separation membrane composed of asymmetric polysulfone (6 mm in diameter) was added to retain unlysed white blood cells and RBC debris (Vivid GR plasma separation membrane, Cytiva). The different layers were secured to each other using double-sided adhesive (Flexmount Select DF021621). Finally, two

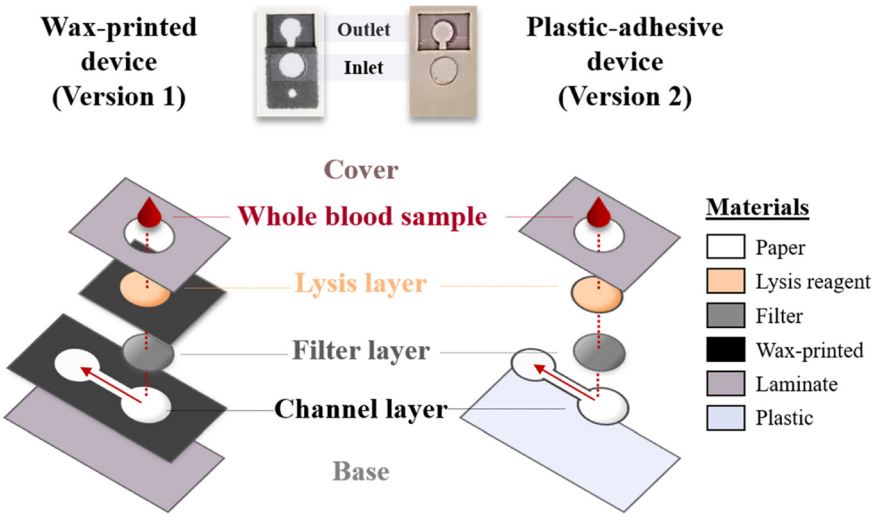


Fig. 1 Lysis device schematic. Exploded schematics of each layer of paper, adhesive and laminate to achieve whole blood lysis using a dried lysis reagent (saponin or Triton X-100). Wax-printed lysis device is described as version 1 and plastic-adhesive lysis device is described as version 2.

transparency laminates (Fellowes and Avery, purchased from Amazon) were placed as cover and base to protect the device from environmental contaminants and user handling.

In **Version 2** of the device (Fig. 1, right), the microfluidic features of the lysis and channel components were defined by laser cutting. In this device, each layer was assembled using double-sided adhesive with openings smaller than the cut paper layers to grant tight sealing and prevent the sample from leaking. To add robustness to the lysis device, the base laminate was also replaced by a 10 mil Mylar sheet (McMaster Carr).

In both cases, after device assembly, the blood lysis layer was modified with a hemolytic reagent. For this, either 7 μL of 50% (w/v) saponin solution or 6 μL of 10% (v/v) Triton X-100 solution (both prepared in ASTM Type I distilled water, Fisher Scientific) were pipetted onto the paper. This procedure produced a 6.5 mm spot with a final concentration of 10.5 mg cm^{-2} and 1.8 mg cm^{-2} of saponin and Triton X-100, respectively. Once the hemolytic reagents were absorbed by the lysis layer, they were dried at 56 °C for 5 minutes.

2.3. Hematocrit measurement

To measure the hematocrit, 3 μL of whole blood was added to a 40 mm microhematocrit capillary tube, and one end was sealed with Critoseal. Then these capillary tubes were processed *via* centrifugation at 1200 rpm for 3 minutes using a ZipCombo centrifuge (LW Scientific). Scanned images of the tubes were obtained using an 8 bit EPSON Perfection V600 PHOTO scanner with a resolution of 800 dpi. The hematocrit of the sample was determined by calculating the ratio between the length occupied by compacted RBCs in the tube and that of the total sample volume.

2.4. Hemoglobin measurement

Total hemoglobin was quantified in whole blood samples before and after paper-based lysis to determine the percentage of hemoglobin recovery and thus the efficiency of paper-based lysis. Briefly, a 5 mm diameter punch was used to retrieve the sample zone from the outlet of the device. This paper disk was agitated on a benchtop orbital shaker for 30 minutes at 300 rpm in 1 mL of Drabkin's reagent with 0.5 μL of Brij 30%. Drabkin's reagent first oxidizes hemoglobin which then reacts with cyanide to generate a very stable compound that can be measured at a single wavelength. Total hemoglobin was estimated by measuring absorbance at 540 nm using a Varioskan LUX microplate reader and the value was interpolated in a calibration plot obtained from a dilution series of hemoglobin standards. Recovered hemoglobin after paper-based blood lysis was compared to pre-lysis whole blood hemoglobin to evaluate the lysis efficiency of the paper devices.

2.5. Detection of malaria biomarkers in *Plasmodium*-infected samples

The concentration of Pf-LDH in samples was detected *via* ELISA after lysing them with the paper-based lysis devices.

2.5.1. *Plasmodium falciparum in vitro* cultures. The *P. falciparum* 3D7 strain was cultured at 37 °C in group B human RBCs (3% hematocrit) following standard protocols.[17] Briefly, parasites were grown in Roswell Park Memorial Institute complete medium containing 5 g L^{-1} AlbuMAX II and 2 mM L-glutamine (RPMI-A, Invitrogen) under a gas mixture of 92.5% N_2, 5.5% CO_2, and 2% O^2. The medium was changed every 1–2 days, and parasitemia was maintained below 5% by dilution with fresh RBCs.

For standard assays, parasitemia of cultures previously synchronized at the ring stage by 5% sorbitol lysis was determined by flow cytometry using a BD LSRFortessaTM cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Samples with parasitemias ranging from 0.0058 to 1.5% (37% hematocrit) were then prepared by serial dilution of the initial culture with fresh RBCs and RPMI-A.

Human blood employed in these cultures was obtained from the *Banc de Sang i Teixits* (https://www.bancsang.net) after being discarded for transfusion, anonymized and irreversibly dissociated from the original database. The study was approved by the Ethical Committee on Clinical Research from the *Hospital Clínic de Barcelona* (Reg. HCB/2018/1223).

2.5.2. Clinical samples. Whole blood samples were obtained before treatment administration from patients attending Vall d'Hebron Hospital with symptoms of malaria. This study had been approved by the Ethics Committee of Vall d'Hebron University Hospital (PR(AG)30/2018) and informed consent was obtained from all participants. Malaria acute infections were confirmed by microscopy and/or by PCR.

Negative control whole blood samples were obtained from Research Blood Components (Watertown, MA, USA). The vendor follows American Association of Blood Banks guidelines for all donors, which includes IRB approved consent to the use of collected blood for research purposes. All research was approved by the Tufts University Institutional Biosafety Committee.

2.5.3. Sample hemolysis. For tube-based lysis, samples were mixed 1 : 1 with a lysis buffer containing 1% Triton X-100 solution for 5 minutes at room temperature. Samples were then centrifuged to pellet cell debris and the supernatants transferred to new tubes. For paper-based lysis, 60 μL of each sample was applied to the inlet of the device. After 10 minutes, all the sample volume had been absorbed and the lysed blood filled the outlet of the paper device, which indicated that the sample was ready to be extracted or stored. To extract hemolysate, the outlet zone was removed using a 5 mm punch and agitated for 30 minutes on an orbital shaker at 300 rpm in 240 μL of PBST–BSA. The extraction was planned to be performed immediately before the ELISA incubation step.

2.5.4. Shortened ELISA for Pf-LDH detection. Unless specified otherwise, all incubations were carried for 1 hour at 37 °C and were followed by three serial washes with PBST. First, the wells of a high-binding plate (Ref. 9018, Corning) were incubated with a solution of 2.5 μg mL^{-1} cMAb in PBS. The plate was then blocked with PBS–BSA. Next, 100 μL of the lysed samples and 10 μL of dMAb (final concentration of 75 ng mL^{-1}) was added into each well and incubated for 30 minutes at 37 °C. The plate was incubated for 10 minutes at room temperature with 50 ng mL^{-1} of PolyHRP before being washed four times with PBST. Finally, 100 μL of TMB substrate solution was added to each well, incubated for 20 minutes, and absorbance was measured at 450 nm after the addition of 50 μL of stop solution (1 M H_2SO_4).

2.6. Data analysis

All experiments were performed at least in duplicate and each paper lysis device produced enough extracted lysed blood for two independent replicates. Accordingly, the points shown in the graphs are the averages of no less than four replicates and the error bars correspond to their standard deviation. The limits of detection (LOD) and quantification (LOQ) of the ELISA were calculated from the averaged signal of the blanks plus 3 and 10 times their standard deviation, respectively. Lysis efficiency was estimated by calculating the ratio between the absorbance measured at 540 nm before and after sample lysis. In the same way, antigen % recovery was calculated by measuring antigen concentration by ELISA before and after lysis.

3. Results and discussion

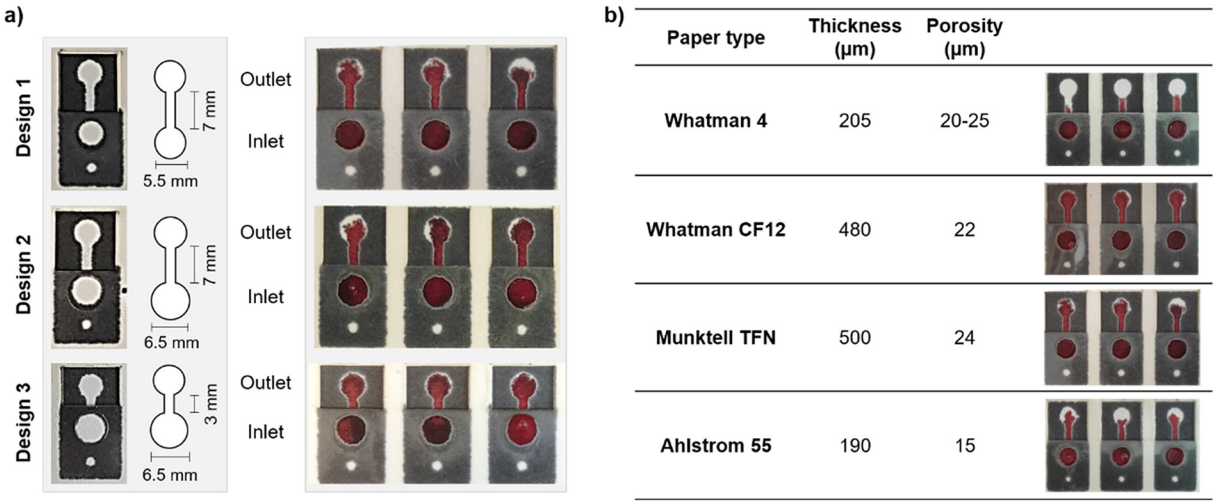
3.1. Design of the lysis device

The first objective in designing these lysis devices was to ensure that a sufficient volume of hemolysate reached the sampling area. To optimize blood flow, different geometries were tested on the wax-printed device with inlets and channels of different dimensions (Fig. 2a). Design 3, which had a larger inlet and shorter channel, was the only design that completely lysed the blood and transferred the hemolysate to the outlet for a 60 μL sample volume.

The paper-based material used to produce the device was also crucial in its performance (Fig. 2b). Four grades of cellulosic filter paper were compared: Munktell TFN (now Ahlstrom TFN), Ahlstrom 55, and Whatman Grades 4 and CF12. For a fixed device geometry (design 1) and volume of blood (60 μL at 40% hematocrit), Munktell TFN and Whatman CF12 more completely filled the outlet with lysed blood. These materials were twice as thick as Whatman Grade 4 and Ahlstrom 55. These data suggested that blood lysis was more efficient in devices that allowed fast blood entry, lysis and transfer to the outlet, which presumably minimized the risk of blood and channel clotting. In the devices made of either Munktell TFN or Whatman CF12 (design 3), an outlet punch of 5 mm in diameter (0.196 cm^2) had a maximal absorption capacity of 12 μL of lysed blood, but Munktell TFN provided more reproducible blood absorption and recovery (Fig. S1a). With this material, 60 μL of sample added to the inlet resulted in complete saturation of the whole device and any excess of sample remained in the inlet without altering the recovered volume in the outlet (Fig. S1b), (see SI Video). This seemed interesting for technology implementation in the field, suggesting that capillary blood could be obtained directly from finger pricks, without having to use pipettes for precise volume measurement.

3.2. Paper-based lysis efficiency

The extent of blood lysis achieved with the two versions of paper-based devices tested was estimated by comparing the concentration of total hemoglobin detected in blood samples before and after paper-based lysis. To test lysis efficiency, each device type was assembled using a lysis layer that was modified with a concentrated hemolytic reagent. Three reagent compositions were compared: 50% saponin, 5% Triton X-100, and a mixture of 25% saponin with 2.5% Triton X-100. These were selected to provide, after sample-driven reconstitution, the minimal concentration of each hemolytic



Paper type	Thickness (μm)	Porosity (μm)
Whatman 4	205	20-25
Whatman CF12	480	22
Munktell TFN	500	24
Ahlstrom 55	190	15

Fig. 2 Optimization of the lysis device geometry and material. (a) Effect of device geometry in the recovery of lysed whole blood (60 μL of blood at 40% hematocrit; wax-printed devices produced on Munktell TFN). (b) Performance of devices produced with different paper-based materials (design 1).

reagent that promoted complete RBC lysis in tube (5% saponin, 0.5% Triton X-100, or a mixture of 25% Triton X-100 and 2.5% saponin; Fig. S2). The general workflow can be seen in Fig. 3a, where the lysis reagents were dried down, blood was applied to devices, and hemoglobin was extracted and analyzed. A calibration curve was made in parallel from freshly-prepared hemoglobin standards (Fig. 3b). In all cases, the concentrations of hemoglobin detected before and after paper-based lysis were comparable, suggesting that paper-based blood lysis and lysate recovery were high.

Two alternative device designs were tested in parallel (Fig. 1). In version 1, based on a previous prototype,[9] the paper-based components were wax-printed to define the microfluidic features. While wax-patterning is a popular approach, the wax-printers used in their fabrication are no longer commercially available, and wax-defined patterns fail to maintain structural integrity in the presence of amphiphilic reagents (*e.g.*, surfactants). Therefore, version 2 was produced, in which the microfluidic structures were fabricated by laser cutting instead. Wax-printed devices demonstrated good performance for lysing whole blood at hematocrits spanning 25–55% when using saponin as the hemolytic reagent (Fig. 3c). However, other lysis reagents, like Triton X-100 (Fig. S2a), a well-known detergent used for cell lysis, protein extraction and viral inactivators,[18,19]

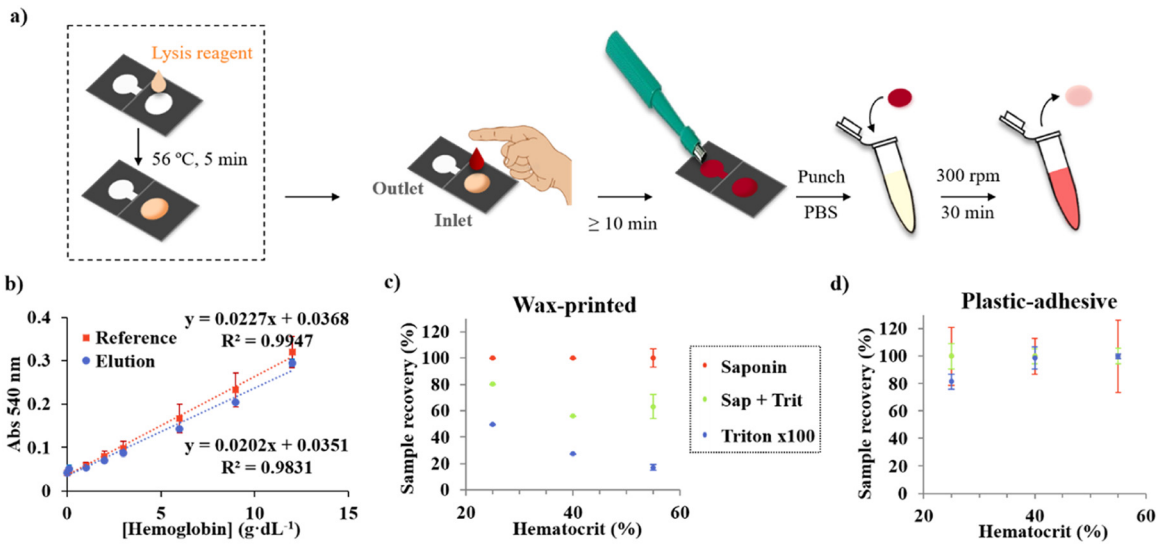


Fig. 3 Whole blood lysis in the paper-based device. a) Schematic summarizing the procedure for the preparation of the lysis device and paper-based whole blood lysis. A concentrated hemolytic reagent was added and dried in the lysis layer. Blood was added to the inlet. After 10 min, the outlet was recovered using a punch and lysed blood was eluted for hemoglobin measurement. b) Detection of increasing concentrations of hemoglobin, measured directly (red) or after drying and extracting from a paper-based lysis device (blue). (c and d) Extent of sample recovery when using different lysis reagents in a wax-printed (c) or a plastic-adhesive lysis device (d).

disrupted the wax barrier causing blood spreading around the inlet (Fig. S3). This disruption resulted in reduced recovery of lysed blood at the outlet, which was more evident for higher hematocrits where blood viscosity is known to be elevated (Fig. 3c).[20] For example, wax-printed devices modified with Triton X-100 displayed RBC lysis efficiency ranging between 20–50%, compared to an average efficiency of 100% displayed by saponin-modified wax-printed devices. In contrast, the plastic-adhesive devices provided complete RBC lysis for the three conditions tested, independent of blood hematocrit, showing the versatility of this new device version (Fig. 3d).

It had been previously reported that blood flow improves at paper devices pretreated with EDTA.[16] Upon optimization, the fast lysis provided by this manuscript's paper-based lysis devices made unnecessary the use of anticoagulants when studying fresh blood. As shown in Fig. S4, whole blood treated with either EDTA or heparin and capillary blood obtained with a lancet without anticoagulant flowed similarly in the devices. Furthermore, hemoglobin quantification revealed complete lysis in all cases. These data suggested that the devices could be used for the study of capillary blood from a pricked finger.

3.3. Detection of malaria biomarkers in paper-lysed whole blood

In order to confirm that RBC lysis was favorable to detect biomarkers of malaria, *P. falciparum* was grown in human RBCs cultured *in vitro*. This culture was then diluted serially with uninfected RBC to produce a range of controlled *P. falciparum* parasitemias at a fixed hematocrit (40%). As illustrated in Fig. 4a, ELISA detection of Pf-LDH, a key enzyme in the parasite anaerobic carbohydrate metabolism, was successful in the cultures, both before and after tube-based RBC lysis. However, detection of Pf-LDH increased up to four times if samples had been lysed. The next step was determining sample lysis efficiency when cultures were lysed in a tube (standard laboratory procedure) and using version 2 devices modified with Triton X-100. Both lysis procedures produced equivalent readouts (Fig. 4b). These results confirmed the convenience of blood sample's lysis for malaria detection and the efficiency of the lysis devices. Under these experimental conditions, the concentration of Pf-LDH detected by ELISA increased proportionally to culture parasitemia in a range spanning 0.001–0.16%, showing saturated plateau signals at higher parasitemias (Fig. 4c and d). The assay displayed theoretical LOD of 0.0002% parasitemia and LOQ of 0.0006% parasitemia in cultures (*i.e.*, prediluted sample; calculated using 3-sigma and 10-sigma values in the graph in Fig. 4c). These corresponded to signals that, interpolated in a calibrate obtained for a serial dilution of recombinant Pf-LDH spiked in non-infected RBC, revealed concentrations of Pf-LDH of approximately 0.004 ng mL^{-1} and 0.012 ng mL^{-1}, respectively. Remarkably, both values were below the LOD of microscopy, 0.001% parasitemia, which is the gold standard for malaria

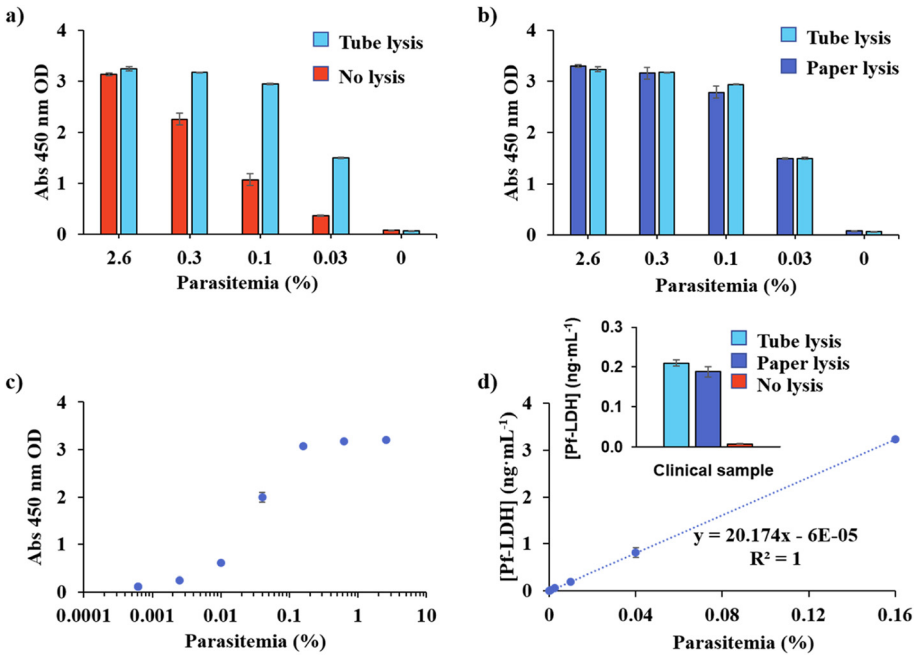


Fig. 4 Detection of Pf-LDH in lysed and unlysed samples. a) ELISA detection of Pf-LDH in human RBCs, infected *in vitro* with decreasing *P. falciparum* parasitemias, before and after tube-based lysis (prediluted % of parasitemia in display). b) Comparison of Pf-LDH detection in these cultures after lysing in a tube or using the paper-based lysis devices. c) Calibration curve for Pf-LDH detection in the cultures after paper-based lysis *versus* parasitemia. d) Correlation between % of parasitemia in the cultures and the concentration of Pf-LDH detected after paper-based lysis (Pf-LDH concentration obtained by interpolating the signal registered for each sample in a calibration curve obtained from a dilution series of recombinant Pf-LDH in the linear range of the assay). The insert summarizes the concentration of Pf-LDH detected by ELISA in a whole blood clinical sample after tube-based lysis, paper-based lysis, and without any lysis at all.

diagnosis.[21,22] Finally, this paper-based lysis device was used to process a clinical sample, which had tested positive for malaria by microscopy. The results revealed that the concentration of Pf-LDH detected by ELISA increased 10 times after blood lysis, independent of the protocol used to process the sample (tube-based or paper-based lysis), suggesting that the paper-based device developed could be applied to the study of clinical samples (Fig. 4d, insert).

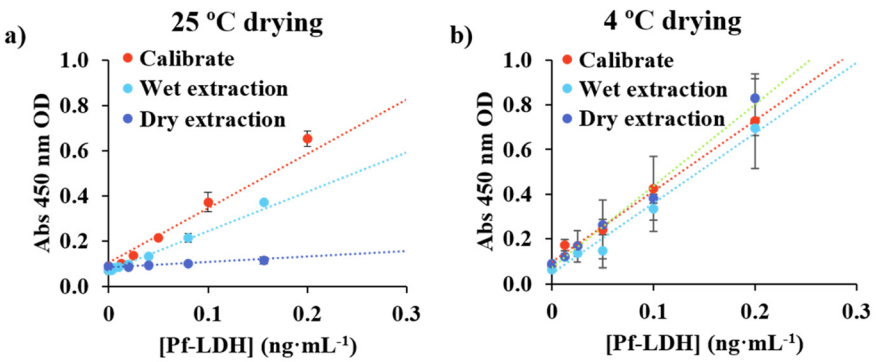
3.4. Stability of paper-based lysis devices as storage cards

The previously described experiments were performed with samples lysed and recovered from the lysis devices the same day they were spotted with blood. The next step was to study if, upon paper-based lysis, samples could be stored in the devices for longer time. The goal was to assess whether devices operated in the field would be suitable for subsequent antigen quantification after transport to a laboratory. With this aim, whole blood was spiked with different concentrations of recombinant Pf-LDH. These samples were then lysed in the paper devices as before, but the devices were next dried at either 25 °C or 4 °C for 1 hour or overnight before extracting the lysed blood and measuring Pf-LDH by ELISA. The results showed that drying at room temperature (25 °C) resulted in a gradual decrease in Pf-LDH detection over storage time. For example, a wet extraction of Pf-LDH (around 1 hour after sample lysis in the paper-based device) resulted in a 25% signal decrease compared to the freshly prepared samples. If they had been stored overnight, the signal was 90% lower than the initial readout (Fig. 5a). In contrast, drying the paper-lysed samples at 4 °C overnight resulted in recovery of antigen concentrations comparable to those in the fresh samples (Fig. 5b).

The study was next extended to longer storage times. Fig. 5c and S5 summarize the stability observed for a range of concentrations of both recombinant Pf-LDH spiked in whole blood and native Pf-LDH in *P. falciparum* grown *in vitro*, after having been dried overnight at 4 °C and then stored at either 4 °C or 25 °C. As it can be seen, paper-dried recombinant Pf-LDH remained stable for up to 1 week only when it was stored at 4 °C. In contrast, the paper-dried native protein could be stored at 25 °C as well. Furthermore, the ELISA revealed recoveries close to 90% after 1 week of storage and above 50% after 1 month of storage, at both temperatures. Tests with azide as a preservative did not showcase any improvement, which indicated that other alternatives should be tested to improve the performance of the paper lysis devices as storage cards at room temperature or for longer times.

4. Conclusions

This work presents the first device capable of automatically lyse whole blood for all human hematocrit levels (25–55%), enabling sample collection and lysis in under 10 minutes. Two device prototypes have been described with different paper-based fabrication methodologies: wax printing and laser cutting. The latter approach allowed whole blood processing using different lysis reagents (saponin and Triton X surfactants) with a very transferable and simple fabrication process. System efficiency was demonstrated using human RBCs infected *in vitro* with *P. falciparum*, as well as a clinical sample from a patient with malaria, which were lysed and analyzed by ELISA to detect Pf-LDH. The paper-based lysis devices successfully lysed all samples, improving 4 times Pf-LDH detection compared to the



c)

Recovery (%)	Recombinant protein 4 °C	Recombinant protein 25 °C	Native protein 4 °C	Native protein 25 °C
24 h	93 ± 13	62 ± 10	90 ± 6	101 ± 5
1 week	103 ± 5	24 ± 3	-	88 ± 21
1 month	44 ± 16	24 ± 6	51 ± 2	53 ± 22

Fig. 5 Stability of paper-dried *Plasmodium* antigens. a and b) Comparison between ELISA detection of a dilution series of recombinant Pf-LDH prepared in tube format (calibrate), or spiked in whole blood, dried in the paper-based lysis devices, and extracted from the outlets. Paper-lysed samples were dried for either one hour (wet extraction) or overnight (dry extraction), at either a) 25 °C or b) 4 °C. c) Percentage recovery of paper-dried recombinant Pf-LDH (spiked in whole blood) and native Pf-LDH (from RBC infected *in vitro* with *P. falciparum*) first dried overnight at 4 °C and then stored at 4 °C or 25 °C for 24 hours, 1 week or 1 month (results on native protein after 1 week at 4 °C were not obtained due to limited volume of fresh sample available).

non-lysed samples. Additionally, paper-based lysis devices were tested as lysed blood storage cards, stabilizing native Pf-LDH for up to 1 week and allowing for 50% recovery after 1 month stored at room temperature. Future work will entail implementing storage strategies to increase the lifespan of the dried lysed blood samples and coupling the lysis device to a rapid diagnostic test.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

Supporting data has been provided as part of the supplementary information (SI). Additional data will be provided upon request.

Supplementary information: Fig. S1, experiments to study device absorption capacity; Fig. S2, effect of different hemolytic reagents in whole blood lysis; Fig. S3, performance of the wax-printed lysis device with different hemolyzing reagents; Fig. S4, anticoagulant dependency; Fig. S5, stability of dried recombinant Pf-LDH and *Plasmodium* parasites; supplementary video, operation and performance of the paper-based device modified (left) or not (right) with a hemolytic reagent. Additional data and videos are available upon request. See DOI: https://doi.org/10.1039/d6lc00145a.

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