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We demonstrate, for the first time, the multiplexed determination of microbial species from whole blood using the paper-folding technique of origami to enable the sequential steps of DNA extraction, loop-mediated isothermal amplification (LAMP), and array-based fluorescence detection. A low-cost handheld flashlight reveals the presence of the final DNA amplicon to the naked eye, providing a “sample-to-answer” diagnosis from a finger-prick volume of human blood, within 45 min, with minimal user intervention. To demonstrate the method, we showed the identification of three species of Plasmodium, analyzing 80 patient samples benchmarked against the gold-standard polymerase chain reaction (PCR) assay in an operator-blinded study. We also show that the test retains its diagnostic accuracy when using stored or fixed reference samples.

Nucleic acid based tests (NATs) offer the promise of microbial diagnostics, determining either the species present or characteristics of the pathogen, such as drug resistance. The gold-standard assay used in many reference laboratories is based upon a polymerase chain reaction (PCR) amplification—a technology which achieves high sensitivities but which also requires trained staff and external power. In contrast, identification of microbial species in resource-limited environments requires low cost, simple tests that do not need external or fixed power supplies. One example where such a simple low-cost test could transform outcomes is in malaria diagnosis, where species identification directly informs patient treatment.

Classical malaria diagnosis involves a blood smear followed by microscopy, which, although simple, does not provide the required sensitivity and only enables species specific information in the hands of trained experts.[5] New approaches will be required to tackle the disease, where asymptomatic individuals commonly harbor the disease at levels that are below the sensitivity of microscopy (< 100 parasites/μL).[2] Nucleic acid based tests (NATs) offer the promise of achieving such high sensitivities (1 parasite/μL) with excellent specificity,[3] enabling healthcare professionals to inform treatment.[4]

As many people living at risk of malaria infection have no access to diagnosis, presumptive treatment of all febrile patients as if they were malaria cases is a common practice, which has become a serious problem, especially in sub-Saharan Africa.[5] Currently, the most widely adopted NAT method in infectious disease diagnosis is still PCR, although the reliance on thermocycling has proven a barrier to its implementation in low-resource settings.[6] LAMP has emerged as a low-cost alternative,[7] simplifying hardware requirements whilst enabling visual detection.[8] Although amenable to multiplexing, the high number of primers required (up to six per target)[9] restricts the number of targets that tests can detect in one reaction. A commercial Plasmodium genus LAMP test is available, but the system requires a multi-step DNA extraction based on the PURE methodology, carried out on a bench-top instrument. Alternatively, lateral flow tests, commonly referred to as “rapid diagnostic tests” (RDT), also exist,[10] but their sensitivity is poor.[11]

Herein, we show a new capillary-flow platform that combines ease-of-use and low-cost with the sensitivity of LAMP, into a multiplexed three taxon-specific test plus a control. We overcome the difficulties linked to sample preparation and multiplexing using capillary wicking and paper-folding origami techniques to distribute fluids both vertically and laterally.[12][13] Previously paper microfluidics (see Review[14]) has enabled single units of a NAT, such as DNA extraction,[15] DNA isothermal amplification,[16] which have been integrated into a manual “machine”[15] with hybridization-based DNA detection.[16] We now integrate all the required steps into a single device to detect Plasmodium falciparum, Plasmodium vivax,[19] and Plasmodium pan directly from a finger-prick volume of whole blood within an operator-blinded study (Figure 1).
Often in-field testing cannot be performed for logistical reasons, and retrospective diagnosis is required. Testing of archived blood samples is also important for epidemiologists to re-visit reference samples or to analyze historical data sets. We therefore show that we can identify parasites in preserved samples of frozen whole blood, as thick and thin fixed smear samples on glass slides, and as whole blood dried onto paper.

The fabrication of the device using wax printing as well as the operating steps, shown in Figure 1 and Figure 2, are all detailed in the Supporting Information. After addition of the sample onto the device (Panel 3, Figure 2A), the paper was folded (structure S1, Figure 2B) to enable the first steps of the assay, involving cell lysis and DNA extraction, to yield purified DNA (Figure 2B–D) on the glass-fiber paper. To transfer the DNA from the extraction panel to the amplification panel, the fold S1 is flipped on the opposite side (Figure 2E), allowing elution (Panels 4–5 of Figure 2A and F). Supporting Information Figure S1(A) illustrates the extraction process.

Multiplexing analysis was enabled by using capillarity to guide the sample to four independent locations on the paper within hot wax printed channels, where species-specific LAMP reagents were deposited (Figure 2A, panel 5). The system was sealed by an acetate film to prevent evaporation during incubation (Figure 1B and D) and amplification was carried out 63°C for up to 45 min on a simple hotplate. The results of species-specific LAMP were initially read-out by the naked eye with a handheld UV lamp (365 nm; E).

The results of species-specific LAMP were initially read-out by the naked eye with a handheld UV lamp (365 nm; E). We also showed that the technique was amenable to quantification (Figure 3B,C). To test sensitivity, we used the WHO International Standard for P. falciparum DNA, which was serially diluted from 10 to 10⁷ times. The real-time amplification curves (Figure 3B) were normalized to 1 for ease of comparison and show that sensitivity down to 10⁵ IU/mL can be achieved within 35 min. As the copy number decreases, so the exponential phase of signal enhancement starts later (Figure 3C) as a consequence of diffusion limited reaction kinetics. For highly infected samples, the time to detection can be significantly faster, down to 12 min for 10⁷ IU/mL.

Our method showed high specificity and good sensitivity for identifying Plasmodium in blood samples (Table 1), when compared against the benchmark PCR. Only for P. falciparum was sensitivity below 80%, as a number of samples for this species were of low parasite density.

All samples were also tested with a commercially-available LAMP kit for malaria (Eiken Chemical Company Ltd. (Japan)), which covers two of our three targets (Plasmodium pan and P. falciparum; Table 2). The specificity for Plas...
The diagnostic accuracy for multiplex-LAMP against reference laboratory diagnosis (using PCR as a benchmark).

| Benchmark PCR         | Origami LAMP | Sensitivity | Specificity |
|-----------------------|--------------|-------------|-------------|
|                       | Positive     | Negative    |             |
| *P. falciparum*       | Pos 11       | Neg 7       | 61%         | 98%         |
|                       | Neg 1        |             |             |             |
| *P. ovale* spp./  *P. malariae* | Pos 27      | Neg 2       | 93%         | 96%         |
|                       | Neg 49       |             |             |             |
| *P. vivax*            | Pos 17       | Neg 1       | 81%         | 98%         |
|                       | Neg 58       |             |             |             |
| Any Plasmodium spp.   | Pos 59       | Neg 10      | 86%         | 100%        |
|                       | Neg 11       |             |             |             |

Plasmodium pan and *P. falciparum* between our method and the LAMP kit, which deploys readout in a commercial turbidim-
or in large cross-sectional studies.[28] As an additional feature of paper-based devices for disease diagnostics, we noted that samples can be readily disposed of by incineration.[29]

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