Ultrasensitive and Robust Point-of-Care Immunoassay for the Detection of *Plasmodium falciparum* Malaria

Teppo Salminen, Farha Mehdi, Deepak Rohila, Manjit Kumar, Sheikh M. Talha,
John Antony Jude Prakash, Navin Khanna, Kim Pettersson, and Gaurav Batra*

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: *Plasmodium falciparum* malaria is widespread in the tropical and subtropical regions of the world. There is ongoing effort to eliminate malaria from endemic regions, and sensitive point-of-care (POC) diagnostic tests are required to support this effort. However, current POC tests are not sufficiently sensitive to detect *P. falciparum* in asymptomatic individuals. After extensive optimization, we have developed a highly sensitive and robust POC test for the detection of *P. falciparum* infection. The test is based on upconverting nanophosphor-based lateral flow (UCNP-LF) immunoassay. The developed UCNP-LF test was validated using whole blood reference panels containing samples at different parasite densities covering eight strains of *P. falciparum* from different geographical areas. The limit of detection was compared to a WHO-prequalified rapid diagnostic test (RDT). The UCNP-LF achieved a detection limit of 0.2−2 parasites/μL, depending on the strain, which is 50- to 250-fold improvement in analytical sensitivity over the conventional RDTs. The developed UCNP-LF is highly stable even at 40 °C for at least 5 months. The extensively optimized UCNP-LF assay is as simple as the conventional malaria RDTs and requires 5 μL of whole blood as sample. Results can be read after 20 min from sample addition, with a simple photoluminescence reader. In the absence of a reader device at the testing site, the strips after running the test can be transported and read at a central location with access to a reader. We have found that the test and control line signals are stable for at least 10 months after running the test. The UCNP-LF has potential for diagnostic testing of both symptomatic and asymptomatic individuals.
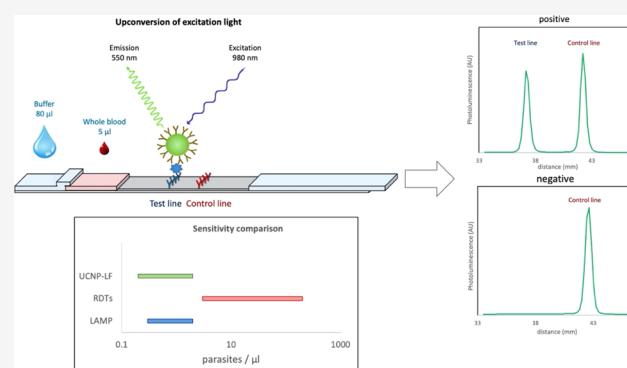
Plasmodium falciparum malaria caused an estimated 405 000 deaths in 2018.[1] Typically, malaria is diagnosed with microscopy or rapid diagnostic tests (RDTs) using the lateral flow (LF) principle. While microscopy is the historical standard method of malaria diagnosis, RDTs are currently the most popular method of diagnosis in resource-poor areas. RDTs are easier to use than microscopy, do not require skilled workers, and produce results quickly. For *P. falciparum* detection with RDTs, the main analyte is *Pf* histidine-rich protein 2, *Pf*HRP2.[2] HRP2 is expressed in the parasite's trophozoite stage, but due to the long half-life of this protein, it can be detected throughout the infection.[3]

There are ongoing efforts to eliminate malaria from large parts of the world, and accurate and rapid diagnosis of low-density infections is a critical component of elimination programs.[4] In the current fight against malaria, rapid diagnostic tests (RDTs) for the detection of malaria parasites are one of the most important components in malaria control campaigns.[5]

A significant challenge for malaria elimination efforts is that malaria infections are often asymptomatic with a low density of parasitemia, which can persist for a year or longer.[6] It has been shown that these asymptomatic individuals may still transmit the parasite to mosquitoes and are an important reservoir for the malaria parasite.[7] Finding and treating these submicroscopic and asymptomatic infections should have a large impact on preventing transmission, since they can account for 20−50% of transmission in preelimination and elimination settings.[8] However, most currently available RDTs do not have sufficient sensitivity to detect asymptomatic or submicroscopic infections where parasite density is below 100 parasites/μL of blood,[9] and many tests even miss samples with a density of 200 parasites/μL.[10] A target product profile for an RDT for the detection of low-density *P. falciparum* infections sets the optimal sensitivity requirement at 10 parasites/μL.[11] An RDT with an even lower sensitivity would be important for malaria elimination programs. According to models, the detection of asymptomatic infections with parasite load below 2 parasites/
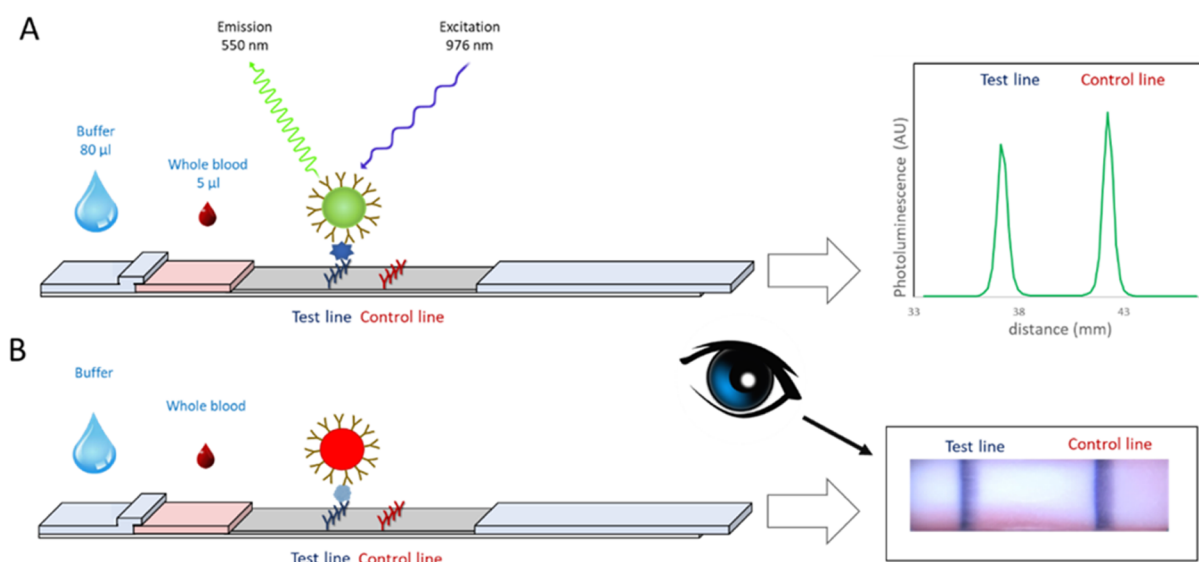
Figure 1. (A) Principle of the UCNP-LF immunoassay for HRP2 antigen detection. The photoluminescence signal of the UCNPs is measured with an instrument by exciting the particles with an infrared laser at a wavelength of 976 nm and measuring the resulting emission at 550 nm. The area containing the test and control lines is scanned, and the line signals are quantified with the instrument. (B) In a conventional LF immunoassay, the label is detected visually by eye.

μL could detect over 95% of the infectious reservoir.[12] This would allow mass-screen-and-treat programs to interrupt transmission in areas with the entomological inoculation rate of over four infectious bites per person per year.[12]

In a typical LF immunoassay, visually detectable nanoparticles coated with tracer antibodies form a sandwich with the analyte and a capture antibody printed on a porous nitrocellulose membrane.[13] To improve the sensitivity of LF immunoassays, fluorescent labels such as Eu nanoparticles[14] and quantum dots[15,16] have been used in place of the conventional visual labels. However, measurement of the long-lifetime fluorescence of Eu nanoparticles requires complex time-gated instrumentation, and quantum dots suffer from high autofluorescence due to the short luminescence lifetime.[16] There are also several other methods to improve the sensitivity of LF immunoassays, but often they involve complex steps to be performed by the end user. These are summarized in the Supporting Information (Supplement 1). In contrast to these complicated methods that require additional steps, in this study, the sensitivity of RDT for the detection of HRP2 antigen was improved using luminescent upconverting nanophosphor (UCNP) reporter particles in place of the typical reflectance-based gold nanoparticle reporter. The use of UCNP reporters allows the development of an ultrasensitive RDT with a test procedure as simple as in the typical RDTs.

In this work, we have developed an RDT that is comparable to traditionally used RDTs in terms of simplicity, hands-on time, total assay duration, sample volume, and does not require any preenrichment or postamplification steps. Despite the simplicity, the UCNP-based RDT provides much higher sensitivity compared to the routinely used HRP2 RDTs that are based on gold or latex nanoparticles (Figure 1).

UCNPs upconvert long-wavelength infrared excitation light into luminescence emission at the visible wavelength, allowing the elimination of sample (including whole blood) and test component autofluorescence background. The UCNP reporters can be used to develop quantitative instrument-read lateral

flow assays with a significant improvement in sensitivity compared to visual lateral flow assays.[17−19]

In this work, we have systematically studied and reported the assay optimization approaches including the strategies for optimal conjugation of UCNP with monoclonal antibody, effect of salt concentration in running buffer on the performance of UCNP-LF, comparison of 12 different nitrocellulose membranes of different wicking rates, comparison of different conjugate pads and optimization of the UCNP conjugate amount on the strip. Very interesting findings on the UCNP flow and membrane wicking rate are reported.

The developed lateral flow assay (UCNP-LF) for PfHRP2 was compared to a WHO-prequalified RDT. Both tests were evaluated with the WHO manufacturer's panel, and the FIND/Zeptometrix panel containing parasite culture samples (eight *P. falciparum* strains) spiked at different densities in whole blood. The instrument-read UCNP-LF test was shown to improve the detection limit approximately 50- to 250-fold compared to the conventional RDT utilizing visually read labels. No improvement in the sensitivity of the conventional RDT was observed when read with a suitable instrument.

■ RESULTS AND DISCUSSION

Lateral flow immunoassays (LFIA) for *P. falciparum* malaria have provided a rapid and convenient method of diagnosing malaria and have helped in reducing malaria cases. Unfortunately, the sensitivity of current LFIA tests is insufficient to detect asymptomatic parasite carriers. We have combined the LFIA format with photoluminescent UCNP labels to develop a highly sensitive yet rapid and robust diagnostic test for *P. falciparum* malaria. The UCNP nanoparticle labels have properties that make them suitable for rapid diagnostic tests. The inorganic particles are stable even at high temperatures, which improves the robustness of RDTs. When the test is measured, the upconversion photoluminescence signal allows the elimination of fluorescence background from the test materials and the sample

matrix, which allows highly sensitive and specific detection of the HRP2 analyte.

UCNP-LF Test Design and Development. We developed a high-sensitivity lateral flow rapid diagnostic test for the detection of *P. falciparum* HRP2 antigen using photoluminescent UCNP labels. The test consists of a plastic backing laminate, nitrocellulose membrane with test and control line stripped 5 mm apart, conjugate pad containing dry tracer antibody−UCNP conjugate, an absorbent pad, and plastic housing for the protection of strip and biocontainment purpose. The transmission electron microscopy image of the unconjugated UCNPs and the size distribution of the particles used in the assay is shown in Supplement 1 (Figure S1). Assay development and optimization included UCNP conjugation with the antibody (Supplement 1, Figure S2), running buffer optimization (Supplement 1, Figure S3), comparison of nitrocellulose membranes of different wicking rates (Supplement 1, Figure S4), comparison of different conjugate pads (Supplement 1, Figure S5), and optimization of the UCNP conjugate amount on the strip (Supplement 1, Figure S6). We conjugated the activated UCNP particles with the tracer antibody in buffers with pH of 6.1, 7.0, and 8.0, and tested each of these conjugates in the UCNP-LF assay for the detection of HRP2 antigen. The conjugate prepared at pH 6.1 had the highest signal-to-background ratio (Supplement 1, Figure S2). The optimal conjugation pH depends on both the properties of the antibody and the EDC/NHS conjugation chemistry. Different concentrations of NaCl in the running buffer was tested, and 500 mM resulted in the highest signal-to-background ratio in the assay (Supplement 1, Figure S3). The ion concentration in the assay influences the antibody binding and the colloidal stability of the UCNP conjugates.

The UCNP-LF strips were assembled with 12 different types of nitrocellulose membranes and compared for the detection of HRP2 antigen. Based on the results, a fast wicking rate nitrocellulose membrane (LFNC-C-SS22_70, Nupore) was chosen to be used in the final assay. Overall, the signal-to-background ratios and signal levels increased with faster wicking rate membranes (Supplement 1, Figure S4). The signal measurement from strips was done at 20 min, which should favor fast wicking rate membranes where the signal from the label is accumulated faster. However, the same trend of higher signal-to-background ratios and higher signal levels was also seen when the different membrane strips were measured the next day after running (data not shown). Thereafter, the UCNP-LF strips were assembled with the chosen nitrocellulose membrane with 10 different conjugate pad materials. Based on the testing in the UCNP-LF assay, the Ahlstrom 8950 (Ahlstrom, Finland) glass fiber conjugate pad was chosen for the final assay (Supplement 1, Figure S5). No correlation was found between signal-to-background ratios and different pad materials or the characteristics provided by the suppliers. In the final development step, the amount of UCNP conjugate was optimized to 10 ng/strip (Supplement 1, Figure S6). A higher amount of UCNP conjugate increase the background signal from the strip resulted in poor signal-to-background ratios.

The sample volume used in the UCNP-LFIA was not optimized but set to 5 μL, since this is the standard sample volume used by malaria RDTs and the goal was to maintain the same assay procedure for the end users that the conventional RDTs use. A higher whole blood sample volume may be feasible for the UCNP-LF, as the background

fluorescence from the whole blood can be eliminated with the upconversion photoluminescence signal.

UCNP-LF Signal Detection. When the UCNP-LF strips are measured with the reader, the resulting signals are calculated by subtracting the baseline signal from both sides of the test line area from the maximum signal achieved at the test line. The subtraction of the baseline signal from the test line peak decreased the variation between the replicates (Figure S7).

Detection Limits. The performance of the optimized UCNP-LF test was compared with a WHO-prequalified conventional RDT that is using colloidal gold nanoparticles as a visual label. Two whole blood panels containing cultured *P. falciparum* from eight different strains were used for evaluation. The panels are available from WHO/CDC and FIND/Zeptometrix and can be used for standardized evaluation of new HRP2 immunoassays. The WHO manufacturer's panel had five strains, and the FIND panel had six strains, with overlapping strains (Tables 1 and 2). These panels cover three types of HRP2 sequences, i.e., types A, B, and C.

Table 1. Analytical Sensitivities of UCNP-LF and First Response HRP2 RDT in pg/mL of HRP2 in FIND Panel

		test analytical sensitivity, pg/mL HRP2		
			First Response HRP2[a]	
sample	HRP2 type	UCNP-LF	visual inspection	mobile-based reader
FCQ79	A	12[b] (400)	400[b] (800)	400[b] (8000)
Benin I	A	12	800	800
Borneo	C	12	400	400
Santa Lucia	B	12	800	800
W2	B	40	800	800
PH1	C	12	800	800

[a]Results for the First Response HRP2 RDT are shown from both visual inspection and as read using mobile-based reader. [b]In-house dilutions, official dilutions in parentheses.

Table 2. Analytical Sensitivities of UCNP-LF and First Response HRP2 RDT in Terms of Parasites/μL in WHO Manufacturer's Panel

		test analytical sensitivity, parasites/μL		
			First Response HRP2[a]	
sample	HRP2 type	UCNP-LF	visual inspection	mobile-based reader
US05F Benin I	A	0.2	50	50
US05F Santa Lucia	B	2	100	100
US08F Nigeria XII	B	0.2	20	20
US05F FC27/A3	B	0.5	10	20
US05F PH1	C	1	50	50

[a]Results for the First Response HRP2 RDT are shown from both visual inspection and as read using mobile-based reader.

For the FIND *P. falciparum* panel, the HRP2 detection limit with the UCNP-LF was found to be 12 pg/mL for five strains and 40 pg/mL for the W2 strain. The detection limit of the conventional RDT was found to be 800 pg/mL for four strains and 400 pg/mL for FCQ79 and Borneo strains. The lowest HRP2 concentrations detected by the tests from dilution series of each sample are shown in Table 1.

The FIND panel sample FCQ79 was tested both with the dilutions provided by the manufacturer and with the same concentrations diluted into whole blood in-house from the provided highest-concentration sample of 8000 pg/mL. This was done because the dilutions provided for FCQ79 strain by the manufacturer gave highly discrepant results compared with the other strains of the panel. The PH1 strain of the same panel was also compared in the same way as in-house dilution control, but there was no difference in the UCNP-LF test results between the manufacturer provided or in-house dilutions for PH1 strain (Supplement 2, Figures S24 and S25).

The WHO manufacturer's panel was provided in two dilutions of 2000 and 200 parasites/μL. Further dilutions were made in-house in whole blood using 2000 parasite/μL samples as starting material. The UCNP-LF achieved a detection limit of 0.2−2 parasites/μL, depending on the strain (Table 2 and Figure 2). The detection limit of the conventional RDT varied
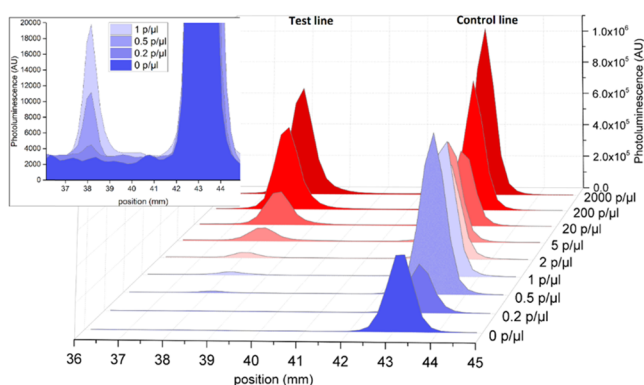
Figure 2. Photoluminescence intensity strip profiles of the UCNP-LF strips, with the WHO manufacturer's panel US05F Benin I sample dilutions as an example. The lowest concentrations tested are also shown in the inset. The X-axis represents the strip position in millimeters, where test line position is approximately 38 mm and control line position is approximately 43 mm. The Y-axis represents the photoluminescence signal from the strip, and the Z-axis represents the concentration of parasites per μL in the samples. The strip profiles of all of the strips are shown in Supplement 2.

from 10 to 100 parasites/μL depending on the strain, when the strips were assessed by the naked eye or by the mobile-based reader (Table 2; Supplement 1, Figure S8; and Supplement 2). There was no significant difference observed in the sensitivity for the conventional RDT when the strips were read by two observers or by the mobile-based reader (Table 2).

The number of replicates and positivity score for all samples on UCNP-LF are shown in Supplement 1, Table S3, and for the commercial RDT with visual assessment in Supplement 1, Table S4. The strip profiles of the UCNP-LF tests and the commercial RDT are shown in Supplement 2. The strip images of the commercial RDT produced by the mobile-based reader are shown in Supplement 3.

UCNP-LF Test Stability and Hook Effect. The prepared UCNP-LF strips remained stable for at least 21 weeks (later time point not tested) at room temperature (22 °C), +40 °C, and +50 °C protected from humidity (Supplement 1, Figure S9). However, the failure rate of the strips stored at +50 °C was 19% (5/27 strips) at the 21-week test point. There were no strip failures after 4 weeks at +50 °C. In the failed strips, the liquids did not flow through the strip and thus no control line was measurable. No failure was observed for the strips stored at

40 °C and room temperature at the 21-week test point. Possible hook effect in the UCNP-LF was tested with recombinant HRP2 Ag up to a concentration of 1 mg/mL, and the test remained clearly positive, showing a wide dynamic range and precluding risks of false negatives due to hook effect (Figure S10).

Comparison with Conventional RDT. The performance of the developed UCNP-LF assay was compared to a WHO-prequalified HRP2 conventional RDT. The results of the conventional RDT with visual labels were read both by the naked eye and with a mobile-based reader. Reading with an instrument did not improve the sensitivity of the visual label RDT. In fact, the sensitivity was slightly poorer with mobile-based reader for some samples due to red background color on some of the test strips interfering with the reading (Table 2).

Jimenez et al. tested four best-in-class WHO-prequalified HRP2 RDTs with three malaria culture strains (Benin I, Santa Lucia, and PH1) and a recombinant HRP2 (W2).[20] The detection limits varied from 400 to 1600 pg/mL of HRP2 in the culture strains and from 800 to 1600 pg/mL with the recombinant HRP2. The tested RDTs were not revealed. The detection limits of the RDTs tested by Jimenez et al. corresponded well to the detection limits of the commercial RDT tested in this study (400−800 pg/mL HRP2, Table 1). Using the same whole blood panel, the analytical sensitivity of the UCNP-LF was 12−40 pg/mL HRP2 (Table 1).

Regarding parasitemia, the detection limit of 0.2−2 parasites/μL achieved with the UCNP-LF test is a 50- to 250-fold improvement over the standard RDT. Microscopy achieves a detection limit of 5−20 parasites/μL in optimal conditions with the gold standard thick film and 50−100 parasites/μL in field conditions.[21,22] Even the commercially available point-of-care targeted nucleic acid amplification test based on loop-mediated isothermal amplification (LAMP) has a detection limit of around 2 parasites/μL.[23]

The developed UCNP-LF uses as binders a very widely used monoclonal antibody pair for HRP2.[24] Therefore, although the sensitivity of different RDTs may be affected by the choice of the binders, it is likely that the dramatic improvement in UCNP-LF test sensitivity compared to existing RDTs is due to the use of the UCNP label in place of the visual labels.

Recently, an ultrasensitive RDT (uRDT) with a *P. falciparum* parasite detection limit of 3 parasites/μL was reported by Das et al.[25] In further studies of the same uRDT in patient populations, the uRDT exhibited a 2-fold increase in detection of infections compared to a standard RDT.[26] The uRDT uses a biotin−streptavidin test line capture system, which may improve the sensitivity of the lateral flow immunoassay by increasing the interaction time between the biotinylated capture antibody and the analyte.[27] However, elevated blood biotin levels from supplement use can cause interference in immunoassays using the biotin−streptavidin system, and therefore, this system was not used in the UCNP-LF.[28] Even though this uRDT shows a major improvement in sensitivity compared to conventional RDTs and can detect previously undetected infections, an even more sensitive rapid test is required for elimination settings. In a recent study of medium-endemic villages, the uRDT detected significantly more infections than the conventional RDT, but still missed 59% of the patients with gametocytes capable of infecting mosquitoes.[29] In contrast, a rapid test with a detection limit of 0.2 parasites/μL should be able to detect over 80% of the infections undetectable by microscopy, which could allow

interruption of the parasite life cycle even in high-endemicity regions.[12] The developed UCNP-LF may serve this need.

Advantages of Upconverting Nanophosphor Labels. The improvement in sensitivity in the LF assay for HRP2 is due to the change of the label from the gold nanoparticles typical for RDTs to UCNPs, as the antibodies used in this work are used in several commercial RDTs but with much lower sensitivity. UCNPs have several advantages as lateral flow immunoassay labels. UCNPs upconvert long-wavelength infrared radiation to shorter wavelength visible light through a multiphoton excitation and long-lifetime phosphorescence emission. Since upconversion is unique to UCNPs, and the anti-Stokes' shift between the excitation and emission wavelength is large with sharp structured emission peaks, there is practically no background photoluminescence from the lateral flow materials or the blood sample. Infrared excitation radiation has an improved penetration of biological materials compared to UV excitation, and UCNP labels have been shown to perform equally in colorless plasma and whole blood. The elimination of autofluorescence background enables highly sensitive detection of the UCNPs.[30]

Unlike visually read gold nanoparticle labels, UCNPs require an instrument for detection. However, a reader for UCNPs can be simple, affordable, and portable. Excitation is done with inexpensive infrared laser diodes, and UCNPs do not suffer from photobleaching so that they can be excited with relatively high energies further improving the potential for high sensitivity.[30] The UCNP-LF strips in this study were measured with a tabletop reader. However, there are several point-of-care UCNP readers described in the literature.[31,32] Moreover, commercial point-of-care UCNP readers have recently become available. The UCNP particles used in this study are synthesized using a solution-phase method, which provides more homogeneous particles compared to particles synthesized by the older solid-state methods.[33] These truly nanosized particles may have better flow properties in the porous nitrocellulose and thus are more suitable for LF assays than the traditional solid-state synthesized upconverting phosphor particles.[30]

Although the described UCNP-LF assay was not developed to produce quantitative results, the wide dynamic range of the UCNPs and the reading of the signal by an instrument could be used to develop a quantitative test of the HRP2 concentration in blood. Quantifying the HRP2 concentration could help discriminate between severe cerebral malaria and other severe illnesses.[34]

Since the UCNP labels are very photostable due to the inorganic crystal lattice of the photoluminescent particle core, the UCNP-LF strips can easily be stored after running the test and measured after long-term storage. The UCNP-LF test can be read at any time after 20 min, also when dried. There was no degradation in the sensitivity or specificity of the test when strips were remeasured 10 months after running the test (Figure 3 and Supplement 2) and after 20 repeated measurements of the same strips (Supplement 1, Figure S11). This may be useful for verification, but more importantly, a test can also be run in a location without access to a reader instrument and the test shipped to be read and archived in a local or regional center with access to the reader. Dried blood spots (DBS) are often used to transport the sample to a laboratory for analysis. In contrast to dried blood spots where the sample is often eluted from the paper and further assay is performed in the central lab, the dried
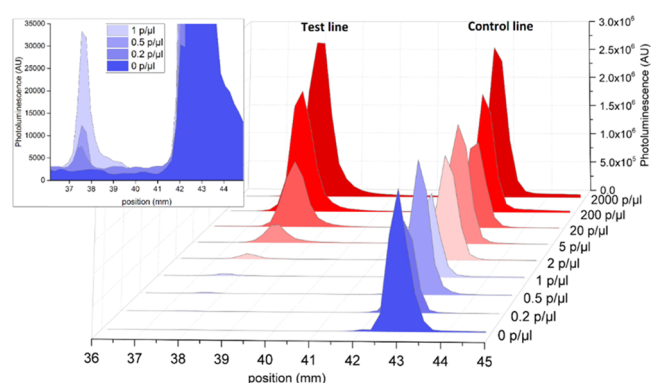
Figure 3. Photoluminescence intensity strip profiles of the UCNP-LF strips measured 10 months after running the test, with the WHO manufacturer's panel US05F Benin I sample dilutions. The lowest concentrations are also shown in the inset. The X-axis represents the strip position in millimeters where test line position is approximately 38 mm and control line position is approximately 43 mm. The Y-axis represents the photoluminescence signal from the strip, and the Z-axis represents the concentration of parasites per μL in the samples. The strip profiles of all of the strips are shown in Supplement 2.

UCNP-LF strips do not need any preparation or elution steps but can simply be read at the central location with access of reader instrument. The UCNP-LF can thus provide simple central laboratory sensitivity testing for areas with only limited access to laboratory instrumentation.

In addition to the improved sensitivity, using a reader has other advantages over visually read RDTs. A reader instrument eliminates the subjectivity involved with the result interpretation. A reader device can easily archive and transmit the result for disease surveillance without the risk of human error.[35]

One limitation of our study is that we have not performed clinical validation of the developed test in the field settings. However, we have checked the appropriateness of the cutoff value with 100 malaria-negative frozen plasma samples, and all scored negative in our assay (data not shown). We also tested 11 microscopy-confirmed *P. falciparum*-positive clinical samples, and all found positive in our assay (additional details in Supplement 1). A multisite clinical performance evaluation study in settings with low parasite density infections is further required to assess the utility of UCNP-LFIA, which may also help to further tune the cutoff value of the assay.

The concept and methodology presented in this work will also be useful for the development of highly sensitive antigen detection assays for other *Plasmodium* targets, e.g., lactate dehydrogenase (pLDH), which would allow the detection of *Plasmodium vivax* infections as well as HRP2 deletion strains of *P. falciparum*.[36] The concept can also be applied for other pathogens. This is particularly important as the highly sensitive molecular tests for the detection of pathogens cannot be applied for large-scale testing for diagnosis and surveillance. Problems with the access and implementation of molecular testing are clearly reflected in the ongoing SARS-CoV-2 pandemic.[37] The UCNP-LF concept may be useful for the development of an ultrasensitive POCT for SARS-CoV-2 antigen detection and could be an alternative of molecular testing.

■ CONCLUSIONS

The sensitivity of a *P. falciparum* HRP2 rapid test can be improved by up to 250-fold, compared to a standard RDT,

using instrument-read UCNP reporters. The resulting assay is as rapid and simple to use as the conventional RDTs, and it could be suitable for detection of low parasitemia asymptomatic patients in elimination or eradication settings. A further improved test would still need to include *Plasmodium* lactate dehydrogenase antigen for the detection of HRP2-deficient strains of *P. falciparum* and detection of other human *Plasmodium* species, especially *P. vivax*.[36] The developed test concept is suitable for highly sensitive detection of antigens from other pathogens with application in routine testing and surveillance.

MATERIALS AND METHODS

Samples and Standards. Human blood was collected in citrate phosphate dextrose adenine anticoagulant-containing tubes (Vacuette CPDA, Greiner Bio-One, Austria). The WHO manufacturer's panel with different cultured *Pf* strains prediluted in whole blood to 2000 and 200 parasites/μL was received from Centers for Disease Control and Prevention, USA. Further dilutions in whole blood were made from 2000 parasites/μL samples. All of the samples were tested with dilutions (in whole blood) corresponding to parasite densities from 0.2 to 2000 parasites/μL. A second panel with cultured malaria strains was purchased from Zeptometrix Corporation (sample product numbers from KZMC041 to KZMC046 and lot numbers from 1611-272-00021 to 1611-272-00026) and was developed by FIND (Foundation for Innovative New Diagnostics, Switzerland) and Zeptometrix. The FIND/Zeptometrix culture panel strains were tested in the dilutions provided by the manufacturer, ranging from 4 to 8000 pg/mL HRP2. Additionally, the FCQ79 and PH1 samples were also tested in the same concentrations by self-made dilutions in whole blood from the highest-concentration samples. Details of the panel samples are shown in Table 1. In addition to culture panels, 11 microscopy-confirmed *P. falciparum*-positive frozen plasma samples and 100 malaria-negative plasma samples were used in this study. These samples were collected after the approval from Translational Health Science and Technology Institute ethics committee (ethics decision no. 1.8.1/(60) dated 03.10.2017) and approval by the ethics committee of Christian Medical College, Vellore (ethics decision no. 10986 dated 22.11.2017). Recombinant HRP2 of type B produced in-house was used for checking the susceptibility of the developed test to the hook effect (prozone effect) and for the stability study.

Bioconjugation of UCNP Reporters. Upconverting nanophosphor (UCNP) particles (Kaivogen, Finland) were used as reporters. The particles had a hydrophilic coating with an average thickness of 10 nm (SD 0.8 nm) and a total diameter of 68 nm (SD 2.7 nm). The particles were conjugated with the anti-HRP2 IgG monoclonal antibody C1-13 (National Bioproducts Institute, South Africa) using EDC-NHS chemistry.

Assembly of the LF Strips. The lateral flow test strips were assembled on a plastic support. The parts assembled onto the support consisted of a 10 mm wide buffer pad, 10 mm wide conjugate pad, 25 mm wide nitrocellulose, and a cellulose absorbent pad. The test line was applied by dispensing 600 ng/cm of PTL3 IgM anti-HRP2 monoclonal antibody (National Bioproducts Institute) with a noncontact dispenser. The control line was dispensed at a 5 mm distance from the test line with 400 ng/cm of polyclonal rabbit anti-mouse immunoglobulins (Dako, Denmark). The UCNP label was dried onto the conjugate pad. The strips were dried for 3 h at +35 °C and stored at room temperature (~22 °C), protected from humidity.

UCNP-LF Procedure. In the UCNP-LF immunoassay, 5 μL of whole blood was applied to the conjugate pad, followed by 80 μL of running buffer to the buffer pad. After 20 min, the test and control line UCNP signals were measured with an Upcon reader device (Labrox, Finland). The test-to-control line ratio was calculated according to a formula that subtracts the strip background from the signal of the test line, as described in the Supporting Information (Supplement 1). The importance of the control line signal, in calculating the result, is summarized in Supplement 1.

The analytical sensitivity is calculated as the lowest concentration of the dilution series of the sample, where all of the three replicates give signals above the cutoff. The cutoff is calculated according to the following equation

$$\text{cutoff} = \text{mean of } R \text{ of negative samples}$$
$$+ 3 \times \text{standard deviation of } R \text{ of negative samples}$$

The cutoff was calculated with a total of 186 replicates tested from the fresh whole blood of eight assumed parasite-negative people as well as the six negative whole blood samples provided with the FIND/Zeptometrix panel. The calculated cutoff test-to-control line ratio was 0.335. The obtained cutoff was subsequently validated with 100 frozen plasma samples from malaria-negative individuals.

The details of conventional RDT, First Response Malaria Antigen test kit (Premier Medical Corporation, India), and the procedure are described in the Supporting Information (Supplement 1).

ASSOCIATED CONTENT

⑤ Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c02748.

Supplement 1: Literature review, materials and methods, and results (PDF)

Supplement 2: Lateral flow strip profiles of UCNP-LF (20 min and 10 months) and colloidal gold-based LF (PDF)

Supplement 3: Photographic results of conventional RDT strips (PDF)

AUTHOR INFORMATION

Corresponding Author
Gaurav Batra − *Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad 121001, Haryana, India;* ⓞ orcid.org/0000-0003-1669-4310; Email: gaurav.batra@thsti.res.in

Authors
Teppo Salminen − *Department of Biotechnology, University of Turku, Turku 20520, Finland*
Farha Mehdi − *Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad 121001, Haryana, India*
Deepak Rohila − *Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad 121001, Haryana, India*

Manjit Kumar − *Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad 121001, Haryana, India*

Sheikh M. Talha − *Department of Biotechnology, University of Turku, Turku 20520, Finland*

John Antony Jude Prakash − *Department of Clinical Microbiology, Christian Medical College, Vellore 632004, Tamil Nadu, India*

Navin Khanna − *International Centre for Genetic Engineering and Biotechnology, New Delhi 110067, India*

Kim Pettersson − *Department of Biotechnology, University of Turku, Turku 20520, Finland*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.analchem.0c02748

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) *World Malaria Report 2019*; World Health Organization: Geneva, 2019.

(2) Mouatcho, J. C.; Dean Goldring, J. P. *J. Med. Microbiol.* **2013**, *62*, 1491−1505.

(3) Marquart, L.; Butterworth, A.; McCarthy, J. S.; Gatton, M. L. *Malar. J.* **2012**, *11*, No. 74.

(4) The malERA Refresh Consultative Panel on Tools for Malaria Elimination. *PLoS Med.* **2017**, *14*, No. e1002455.

(5) Pham, N. M.; Karlen, W.; Beck, H. P.; Delamarche, E. *Malar. J.* **2018**, *17*, No. 260.

(6) Bousema, T.; Okell, L.; Felger, I.; Drakeley, C. *Nat. Rev. Microbiol.* **2014**, *12*, 833−840.

(7) Churcher, T. S.; Bousema, T.; Walker, M.; Drakeley, C.; Schneider, P.; Ouédraogo, A. L.; Basáñez, M. G. *eLife* **2013**, *2*, No. e00626.

(8) Galatas, B.; Bassat, Q.; Mayor, A. *Trends Parasitol.* **2016**, *32*, 296−308.

(9) Girma, S.; Cheaveau, J.; Mohon, A. N.; Marasinghe, D.; Legese, R.; Balasingam, N.; Abera, A.; Feleke, S. M.; Golassa, L.; Pillai, D. R. *Clin. Infect. Dis.* **2019**, *69*, 1003−1010.

(10) World Health Organization. *Malaria Rapid Diagnostic Test Performance. Results of WHO Product Testing of Malaria RDTs: Round 7 (2015−2016)*; 2017.

(11) Program for Appropriate Technology in Health; PATH. *Target Product Profile: Point-of-Care Malaria Infection Detection Test For Rapid Detection of Low-Density, Subclinical Malaria Infections*, 2014; p 45.

(12) Slater, H. C.; Ross, A.; Ouédraogo, A. L.; White, L. J.; Nguon, C.; Walker, P. G. T.; Ngor, P.; Aguas, R.; Silal, S. P.; Dondorp, A. M.; La Barre, P.; Burton, R.; Sauerwein, R. W.; Drakeley, C.; Smith, T. A.; Bousema, T.; Ghani, A. C. *Nature* **2015**, *528*, S94−S101.

(13) Posthuma-Trumpie, G. A.; Korf, J.; Van Amerongen, A. *Anal. Bioanal. Chem.* **2009**, *393*, 569−582.

(14) Salminen, T.; Juntunen, E.; Talha, S. M.; Pettersson, K. *J. Immunol. Methods* **2019**, *465*, 39−44.

(15) Di Nardo, F.; Anfossi, L.; Giovannoli, C.; Passini, C.; Goftman, V. V.; Goryacheva, I. Y.; Baggiani, C. *Talanta* **2016**, *150*, 463−468.

(16) Foubert, A.; Beloglazova, N. V.; De Saeger, S. *Anal. Chim. Acta* **2017**, *955*, 48−57.

(17) Corstjens, P. L. A. M.; Van Lieshout, L.; Zuiderwijk, M.; Kornelis, D.; Tanke, H. J.; Deelder, A. M.; Van Dam, G. J. *J. Clin. Microbiol.* **2008**, *46*, 171−176.

(18) van Dam, G. J.; de Dood, C. J.; Lewis, M.; Deelder, A. M.; van Lieshout, L.; Tanke, H. J.; van Rooyen, L. H.; Corstjens, P. L. A. M. *Exp. Parasitol.* **2013**, *135*, 274−282.

(19) Juntunen, E.; Arppe, R.; Kalliomäki, L.; Salminen, T.; Talha, S. M.; Myyryläinen, T.; Soukka, T.; Pettersson, K. *Anal. Biochem.* **2016**, *492*, 13−20.

(20) Jimenez, A.; Rees-Channer, R. R.; Perera, R.; Gamboa, D.; Chiodini, P. L.; González, I. J.; Mayor, A.; Ding, X. C. *Malar. J.* **2017**, *16*, No. 128.

(21) Ochola, L. B.; Vounatsou, P.; Smith, T.; Mabaso, M. L. H.; Newton, C. R. J. C. *Lancet Infect. Dis.* **2006**, *6*, 582−588.

(22) Wongsrichanalai, C.; Barcus, M. J.; Muth, S.; Sutamihardja, A.; Wernsdorfer, W. H. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 119−127.

(23) De Koninck, A. S.; Cnops, L.; Hofmans, M.; Jacobs, J.; Van Den Bossche, D.; Philippé, J. *Malar. J.* **2017**, *16*, No. 418.

(24) UNITAID. *Malaria Diagnostics Technology and Market Landscape*, 3rd ed.; Unitaid Secretariat World Health Organization, 2016; Vol. 28, pp 1−148.

(25) Das, S.; Peck, R. B.; Barney, R.; Jang, I. K.; Kahn, M.; Zhu, M.; Domingo, G. J. *Malar. J.* **2018**, *17*, No. 118.

(26) Das, S.; Jang, I. K.; Barney, B.; Peck, R.; Rek, J. C.; Arinaitwe, E.; Adrama, H.; Murphy, M.; Imwong, M.; Ling, C. L.; Proux, S.; Haohankhunnatham, W.; Rist, M.; Seilie, A. M.; Hanron, A.; Daza, G.; Chang, M.; Nakamura, T.; Kalnoky, M.; Labarre, P.; Murphy, S. C.; McCarthy, J. S.; Nosten, F.; Greenhouse, B.; Allauzen, S.; Domingo, G. J. *Am. J. Trop. Med. Hyg.* **2017**, *97*, 1540−1550.

(27) Holstein, C. A.; Chevalier, A.; Bennett, S.; Anderson, C. E.; Keniston, K.; Olsen, C.; Li, B.; Bales, B.; Moore, D. R.; Fu, E.; Baker, D.; Yager, P. *Anal. Bioanal. Chem.* **2016**, *408*, 1335−1346.

(28) Bayart, J. L.; Favresse, J.; Stoefs, A.; Closset, M.; Roy, T.; Fillée, C.; Rodriguez-Villalobos, H.; Kabamba-Mukadi, B.; Gruson, D. *Clin. Chim. Acta* **2020**, *503*, 107−112.

(29) Hofmann, N. E.; Gruenberg, M.; Nate, E.; Ura, A.; Rodriguez-Rodriguez, D.; Salib, M.; Mueller, I.; Smith, T. A.; Laman, M.; Robinson, L. J.; Felger, I. *Lancet Infect. Dis.* **2018**, *18*, 1108−1116.

(30) Riuttamäki, T.; Soukka, T. *Bioanal. Rev.* **2014**, *1*, 155−204.

(31) Yang, X.; Liu, L.; Hao, Q.; Zou, D.; Zhang, X.; Zhang, L.; Li, H.; Qiao, Y.; Zhao, H.; Zhou, L. *PLoS One* **2017**, *12*, No. e0171376.

(32) You, M.; Lin, M.; Gong, Y.; Wang, S.; Li, A.; Ji, L.; Zhao, H.; Ling, K.; Wen, T.; Huang, Y.; Gao, D.; Ma, Q.; Wang, T.; Ma, A.; Li, X.; Xu, F. *ACS Nano* **2017**, *11*, 6261−6270.

(33) Haase, M.; Schäfer, H. *Angew. Chem., Int. Ed.* **2011**, *50*, 5808−5829.

(34) Hendriksen, I. C. E.; White, L. J.; Veenemans, J.; Mtove, G.; Woodrow, C.; Amos, B.; Saiwaew, S.; Gesase, S.; Nadjm, B.; Silamut, K.; Joseph, S.; Chotivanich, K.; Day, N. P. J.; Von Seidlein, L.; Verhoef, H.; Reyburn, H.; White, N. J.; Dondorp, A. M. *J. Infect. Dis.* **2013**, *207*, 351−361.

(35) Kozel, T. R.; Burnham-Marusich, A. R. *J. Clin. Microbiol.* **2017**, *55*, 2313−2320.

(36) Thomson, R.; Parr, J. B.; Cheng, Q.; Chenet, S.; Perkins, M.; Cunningham, J. *Bull. World Health Organ.* **2020**, *98*, 558−568.

(37) Cheng, M. P.; Papenburg, J.; Desjardins, M.; Kanjilal, S.; Quach, C.; Libman, M.; Dittrich, S.; Yansouni, C. P. *Ann. Intern. Med.* **2020**, *172*, 726−734.