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Low-Resource Method for Extracting the Malarial Biomarker Histidine-Rich Protein II To Enhance Diagnostic Test Performance

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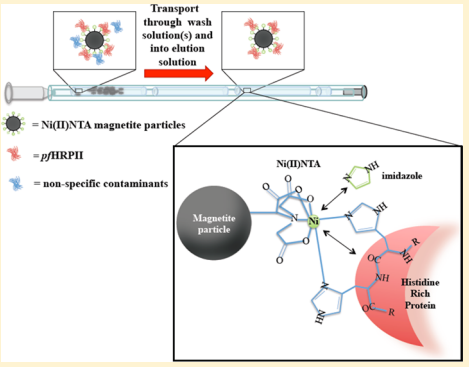
Supporting Information

ABSTRACT: We have demonstrated the utility of a self-contained extraction device for the selective isolation, purification, and concentration of the malaria diagnostic protein biomarker *Plasmodium falciparum* histidine-rich protein II (*pf*HRPII) from human plasma and whole blood. The extraction cassette consists of a small-diameter tube containing a series of preloaded processing solutions separated by mineral oil valves. Nickel(II) nitrilotriacetic acid-functionalized magnetic particles are added to a parasite-spiked sample contained within the loading chamber of the device for capture of *pf*HRPII. The biomarker-bound magnetic particles are then entrained by an external magnetic field and transported through three wash solutions. Processing removes sample interfering agents, and the biomarker target is concentrated in the final chamber for subsequent analysis. At parasitemias of 200 parasites/μL, purification and concentration of *pf*HRPII with extraction efficiencies in excess of 70% total protein target are achieved. The concentration of nonspecific protein interfering agents was reduced by more than 2 orders of magnitude in the final extracted sample without the need for hours of processing time and specialized laboratory equipment. We have demonstrated an application of this low-resource technology by coupling extraction and concentration of *pf*HRPII within the cassette to a commonly employed rapid diagnostic test. Sample preprocessing improved the visual limit of detection of this test by over 8-fold, suggesting that the combination of both low-resource technologies could prove to be useful in malaria eradication efforts.



Protein biomarkers are important targets in many immunochromatic rapid diagnostic tests (RDTs).[1,2] These tests offer many advantages, including ease of use, applicability to a broad range of medical conditions, rapid development time for relatively small investment, and low cost to the patient. For these reasons, RDTs have become important diagnostic tools in low-resource settings. Unfortunately, RDT efficacy is often limited by sample condition and purity, target concentration, environmental conditions, and antibody failures.[3,4] Many point-of-care (POC) diagnostics are only modest in sensitivity, have a limited dynamic range, and are not quantitative. In a recent World Health Organization (WHO) comparative analysis of WHO-qualified RDTs for malaria infection, 26% of the RDTs tested had detection rates above 90% at the WHO-recommended lower limit of detection, 200 parasites/μL.[5] Such levels of parasitemia are clinically important for patients from nonendemic regions and in cases of asymptomatic malaria in which the patient serves as a transmission reservoir. The design of a simple device that purifies and concentrates a protein biomarker in a low-resource setting could significantly improve accurate diagnosis using standard RDTs.

Current fluidic approaches for the concentration and purification of protein biomarkers include microfluidic and paper devices.[6−8] Microfluidic devices aim to provide multistep sample-processing and detection procedures in a small, disposable all-in-one device.[9,10] Incorporation of immunoassays into micrometer-sized channels allows for smaller sample and reagent volumes as well as shorter test times,[11,12] but mechanical failure of valves and pumps and cost of manufacturing and materials impose limitations on the availability of such devices in low-resource areas.[13] Paper-based diagnostics were developed to circumvent the cost and need for valves and pumps in microfluidic cassettes by directing lateral flow of liquids within micrometer-sized channels formed by wax barriers across two- and three-dimensional paper strips and pads.[14,15] However, inconsistencies associated with retention factors of different fluids and samples, nonspecific interfering agents, and paper's susceptibility to environmental conditions can yield failed tests. As an alternative to the valve complexity of traditional microfluidic devices, researchers have developed a rapid purification method based on immiscible phase filtration, where a barrier is created using oil, wax, or organic solvent to separate two aqueous solutions on either the macro- or microscale. This approach utilizes a magnet to transport analyte-bound magnetic particles across the barrier from one solution to the next. This technique has been effective

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in the rapid isolation of nucleic acids,[16,17] proteins,[18,19] and cells.[20,21] Similarly, Haselton and co-workers demonstrated the use of stable air gap barriers between prearranged processing solutions in a filament-based design for the detection of viruses.[22] On the basis of these principles, we have recently reported the development of a self-contained extraction cassette for the isolation of a viral RNA biomarker from nasal washes.[23]

Here, we modify this self-contained design and demonstrate its utility for the extraction of a protein biomarker from a more complex sample matrix, e.g., whole blood. The extraction cassette incorporates preloaded buffer solutions separated by small volumes of mineral oil, which we call surface tension valves, into a single length of tubing. By confining immiscible materials, such as buffers and oil, into a small tube, the interfacial tension at their surface creates a valve which prevents intermixing of contiguous processing solutions but permits the passage of magnetic particles. In proof of concept experiments, the malarial protein biomarker *Plasmodium falciparum* histidine-rich protein II (*pf*HRPII) was extracted from spiked human plasma and whole blood. *pf*HRPII, a 67 kDa protein, contains multiple repeats of AHH and AHHAAD amino acid motifs, which comprise ~85% of the total amino acids in the protein.[24,25] In this paper, we describe the components and efficiency of the cassette, compare its performance to that of lab-based commercial kits, and measure its impact on lowering the limit of detection of currently available RDTs.

EXPERIMENTAL SECTION

_pf_HRPII Extraction from Plasma and Whole Blood Using the Self-Contained Extraction Device. The extraction device and its chamber contents shown in Figure 1

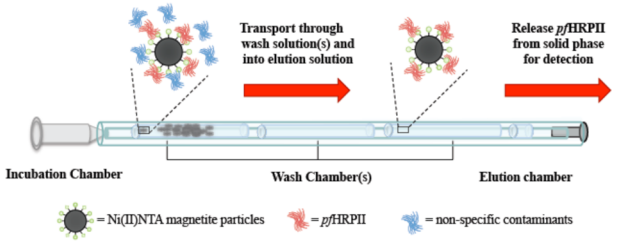

Figure 1. Design and chamber contents of the self-contained *pf*HRPII extraction cassette. Magnetic particles with bound biomarker are pulled through the device with an external magnet.

were prepared by preloading four processing solutions and four mineral oil valves within Tygon R-3603 tubing ~24 cm in length (1.6 mm inner diameter). The rounded end of a 200 μL polymerase chain reaction (PCR) Eppendorf tube was cut, and the top of the cap was punctured with a $27^1/_2$ gauge needle to serve as an air release valve, prior to insertion into one end of the tubing. A 10 μL volume of elution buffer (50 mM potassium phosphate (PB), pH 8.0, 300 mM NaCl, 500 mM imidazole, 0.05% Tween-20) was pipetted into the end of the tubing opposite the PCR tube using a gel tip pipet tip. The elution end of the tube was sealed with the rounded end of a MelTemp capillary tube. Using a $27^1/_2$ gauge syringe, mineral oil (25 μL, ~2 mm length) was added in front of the elution chamber prior to the sequential addition of three 100 μL wash chambers (50 mM PB, pH 8.0, 300 mM NaCl, 125 mM imidazole, and 0.05% Tween 20) separated by mineral oil valves. Mineral oil was also added between the first wash chamber and the end of the PCR tube to seal off the tubing to air. A 200 μL sample of prepared plasma or whole blood was pipetted into the PCR tube, followed by a 10 μL suspension of magnetic Qiagen nickel nitrilotriacetic acid (Ni^{II}NTA) agarose particles (concentration 152.8 particles/μL). Four extraction tubes were prepared for each concentration of parasite spiked into plasma or whole blood. The tubes were incubated on a benchtop rotisserie for 10 min to ensure mixing of the agarose particles throughout the sample. After the incubation period, by threading the tube through the center of a donut magnet (2.5 mm o.d., 1.4 mm i.d., 0.6 mm thickness, 250−300 mT from the center), the particles were collected, pulled across the oil valve, and transferred into the first wash chamber. The particles were mixed with the magnet throughout the first wash chamber for 30 s using a back and forth movement. After mixing, the particles were collected at the end of the wash chamber and pulled across the oil valve into the second wash chamber. Likewise, the particles were mixed within the second and third wash chambers until reaching the final 10 μL elution chamber. In the elution chamber, the particles were mixed for 10 min to ensure total release of the protein from the surface of the particles. After the elution period, the particles were transferred back into the previous oil valve.

Malaria Rapid Diagnostic Test Enhancement. For each concentration of parasite extracted from plasma and whole blood, the 10 μL elution chamber, from a previously prepared extraction tube, was excised, spotted onto an RDT, developed for 20 min (per the manufacturer's specifications), and photographed.

RESULTS AND DISCUSSION

Development and Optimization of the _pf_HRPII Extraction Cassette. The cassette previously designed for the extraction of viral RNA from nasal wash samples incorporated processing chambers separated by air valves within a single length of flexible Tygon tubing.[23] RNA was extracted from the sample via adsorption to the surface of 1 μm magnetic silica particles and processed through the chambers and air valves using an external magnet. In transition from extraction of nucleic acids to protein targets, several parameters of the device were redesigned to make this approach suitable for targeting *pf*HRPII, including selective binding of pfHRPII, incorporation of mineral oil valves, arrangement and composition of the processing chambers, and optimization of operational parameters.

The cassette consists of five sequential aqueous buffer chambers separated by mineral oil valves contained within a single length of flexible Tygon tubing: a binding chamber, three wash chambers, and a final elution chamber (Figure 1). As the 50 μm Ni^{II}NTA agarose particles in this design are significantly larger than the 1 μm particles previously used and are unable to traverse air efficiently, mineral oil was substituted for air. It has been shown that immiscible oil barriers are resistant to deformation within a microfluidic device, due to the interfacial energy at their contact region, which enhances the stability chamber configuration.[17] During the extraction process, the particles are magnetically entrained at the end of a chamber where they are collectively passed through the valve. Once the particles traverse the oil into the next chamber, the mineral oil valve returns to its resting state (Figure S-1, Supporting Information).

Ni^{II}NTA technology has been shown to selectively bind and purify histidine-rich proteins from blood and plasma samples due to selective coordination of the imidazole side chains of

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histidine to the metal center, and thus 50 μm magnetic Ni^{II}NTA agarose particles were selected as the motive solid phase for biomarker capture (Figure S-2, Supporting Information).[24,26,27] Multiple coordination interactions to the nickel metal center are more robust than surface adsorption and ensure that the biomarker is not leached from the surface of the particle. Optimal target binding and elution profiles were determined from kinetic studies. Finding a balance between the time required to capture and release maximum biomarker and the total extraction time was necessary for creation of a rapid and efficient cassette. After 10 min, more than 70% of the biomarker was bound to the particles (Figure 2A). At 30 min,

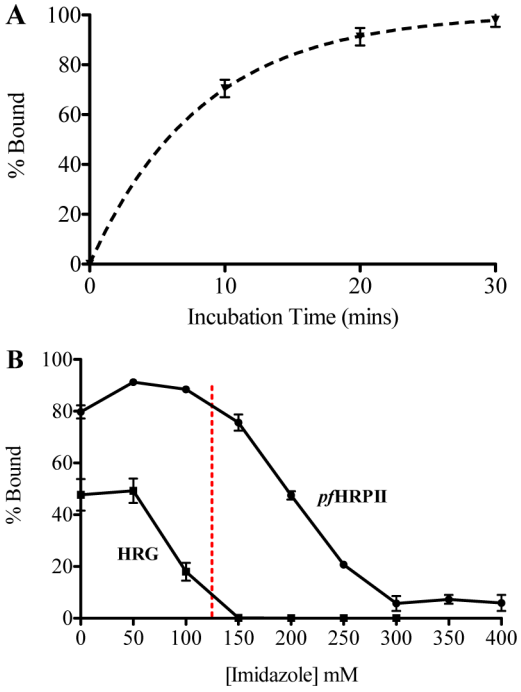

Figure 2. (A) Binding of *pf*HRPII to NiNTA agarose microparticles as a function of time. The rate constant was calculated to be 0.1160 min^{-1} (one phase association model). (B) Binding of *pf*HRPII and HRG to Ni(II)NTA agarose microparticles as a function of varying concentrations of imidazole. Vertical dotted red line indicates the concentration of imidazole chosen for the extraction experiments. Concentration of Ni(II)NTA particles was held constant (152.8 particles/μL).

greater than 90% recovery could be achieved. While the incubation time was ultimately optimized at 10 min, this does not negate the future possibility of increasing the biomarker-binding time to achieve higher yields. The elution time was similarly optimized at 10 min (Figure S-3, Supporting Information).

With the capture of *pf*HRPII in the binding chamber, contaminants, such as blood proteins and cellular debris, must be washed away from the surface of the Ni^{II}NTA particles without interfering with the bound biomarker. Analysis of extraction efficiencies demonstrates that several small volumes are usually more effective at partitioning the analyte into the extraction phase rather than a single large volume.[28] Consequently, three wash chambers were incorporated into the cassette design to serve as isolated purification reservoirs. One of the consequences of the transit of these particles through the mineral oil valve is the entrainment of a small volume of liquid associated with the surface of the particles. This carryover volume was determined to be 1.24 ± 0.56 μL, regardless of the chamber environment (e.g., moving from binding to wash, wash to wash, or wash to elution). Thus, any amount of plasma or whole blood carried over from the loading chamber in this volume will be sequentially diluted 100-fold in each of the subsequent wash chambers.

While isolation of the wash chambers from the binding chamber via mineral oil limits the effect of carryover of contaminating proteins, introducing imidazole to the aqueous phase enhances the level of purification. Patient blood samples contain a host of potential contaminating proteins, such as histidine-rich glycoprotein (HRG) and human serum albumin (HSA), that can interact with the Ni^{II}NTA particles by either specific coordination to the metal ligand or nonspecific association with the bead. HRG, a globular protein with a physiological concentration of 1.5 μM, is characterized by a centralized histidine-rich region with five tandem repeats of GHHPH amino acid motifs and has been shown to chelate free metal ions such as Zn^{2+}.[29−31] Although HRG can coordinate to the metal center, the coordination is weaker than that of *pf*HRPII due to the lower number of metal-binding sites. Therefore, imidazole can be used to competitively block HRG from binding Ni^{II}NTA without disrupting bound *pf*HRPII. Aside from blocking potential metal-coordinating contaminants, imidazole also mediates nonspecific protein interactions (e.g., electrostatic interactions of HSA and hemoglobin (Hb), and other non-metal-coordinating proteins) by charge neutralization of the particle surface.

The imidazole step-gradient elution profile of *pf*HRPII and HRG from spiked plasma is shown in Figure 2B. At an imidazole concentration of 300 mM, *pf*HRPII was completely eluted from the particles, while HRG was released with 150 mM imidazole. Similar observations in the elution profile of *pf*HRPII and HRG were previously noted in purification of *pf*HRPII by Panton et al. using Zn^{2+} affinity chromatography.[24] An overlay of the titration curves of *pf*HRPII and HRG identifies a region between 100 and 200 mM imidazole where the yield and purity of the final sample is maximized. For the sake of maintaining high yields of biomarker and minimizing the presence of contaminants, the blocking conditions in the system were set at 125 mM imidazole (Figure 2B, dotted vertical red line). Global analysis of the purification of target was qualitatively monitored by Coomassie-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels (Figure S-4, Supporting Information).

After the biomarker-bound particles are processed through the wash chambers, *pf*HRPII is released from the surface of the particles into an elution chamber containing 500 mM imidazole. The final chamber volume was minimized to concentrate *pf*HRPII in the final chamber. Optimization experiments determined that a 10 μL elution volume was the minimum convenient volume for protein release (Figure S-5, Supporting Information), resulting in a 10-fold concentration of target.

Specific capture of the malarial protein *pf*HRPII by Ni^{II}NTA magnetic particles and purification of the biomarker from whole blood in a low-resource format distinguish this device from other protein extraction methods. Separation of the binding and elution chambers by three wash chambers limits the carryover of small volumes of contaminants from the original sample, while the composition of the aqueous wash solutions facilitates the removal of interfering agents from the biomarker-

bound particles. The binding and release kinetics of the protein from Ni^{II}NTA ensured that the total extraction time was approximately 25 min, compared with commercial kits, which can have an extraction time of 1.5 h and require several centrifugation and transfer steps. These optimized conditions were subsequently used for the extraction of target biomarker from the complex biological matrixes of plasma and whole blood.

Efficiency of *pf*HRPII Extraction Using the Self-Contained Cassette. The efficacy of the optimized cassette for the extraction of *pf*HRPII from complex matrixes was investigated. Initial studies were conducted on plasma samples spiked with 200 parasites/μL of *pf*HRPII culture. Enzyme-linked immunosorbent assay (ELISA) analysis of all of the chambers demonstrated total recovery (112.4 ± 10.8%) of *pf*HPRII within the cassette. Under these conditions, 74.0 ± 9.1% of the protein was recovered in the elution, and the wash chambers were virtually free of protein (Figure 3, black bars).

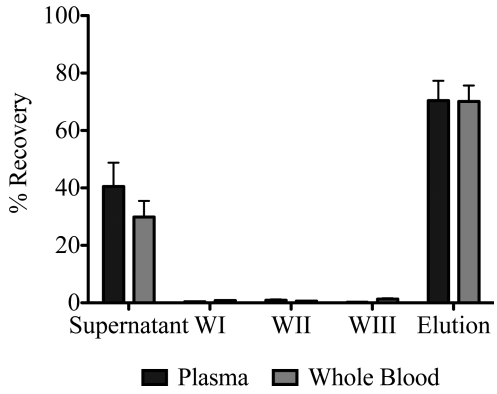

Figure 3. ELISA analysis of the postextraction distribution of *pf*HRPII within the extraction cassette chambers. Biomarker was extracted from spiked plasma (black bars) and whole blood (gray bars). Both samples were spiked to contain 200 parasites/μL (n = 6, mean ± SD). WI, WII, and WIII represent the first, second, and third wash chambers, respectively.

The balance remained unbound in the initial binding chamber. While high yields were achieved from a plasma sample, blood remains an attractive choice for biomarker detection.[32]

Whole blood samples were similarly spiked with 200 parasites/μL of *pf*HRPII culture and processed through the extraction cassette. Preprocessing the lysed blood sample through glass wool eliminated excess cellular debris. As seen with plasma, all (102.7 ± 7.9%) of the *pf*HRPII was accounted for among the chambers of the extraction cassette (Figure 3, gray bars). A total of 70.1 ± 5.6% of the protein was recovered in the elution. Analysis of the results by an unpaired t test showed that there was no significant difference ($p \geq 0.05$) between the recoveries of *pf*HRPII from plasma and whole blood. Additionally, there was no statistical difference ($p \geq 0.05$) in *pf*HRPII recovery from plasma between extractions using a HisPur Spin Column commercial kit (68.8 ± 5.4%), which uses conventional Ni^{II}NTA agarose resin, and extractions using the cassette design. However, recovery of *pf*HRPII from whole blood using the spin kit (45.3 ± 2.1%) versus the extraction cassette was determined to be statistically different (Figure S-6, Supporting Information). The extraction cassette represents an attractive design for low-resource applications, since it achieves the same recovery as laboratory-based methods that require multiple centrifugation/transfer steps and extensive processing time.

The extraction cassette design offers several advantages over recently developed approaches. For example, Stayton and co-workers used stimulus-responsive antibody–polymer conjugates and membranes to isolate and concentrate *pf*HRPII from spiked plasma. The efficiencies of their system ranged from 7% to 35%.[33] The antibody–antigen complex was captured on the membrane at elevated temperatures and released for detection upon cooling. Another assay reported 100% recoveries of *pf*HRPII from 1/5-diluted serum with antibody-conjugated magnetic nanoparticles, but took no steps to remove contaminants.[34] Neither of these systems attempted isolation from whole blood. While both of these approaches are intriguing, they do not sufficiently address the well-established challenges of antibody thermal stability and long-term storage that are also the primary pitfalls of currently available malaria RDTs.[35]

Purification of *pf*HRPII from Spiked Human Plasma and Whole Blood. In conjunction with analyzing the efficiency of *pf*HRPII extraction, purification of *pf*HRPII was also investigated. While the Ni^{II}NTA ligand acts to selectively capture the malarial biomarker, other metal-chelating proteins will compete for binding to the metal center. Because of its propensity toward chelating metal centers, the HRG content was quantified across the extraction cassette by ELISA (Figure S-7, Supporting Information). If the contaminants are coordinated to Ni^{II}NTA, the presence of imidazole in the incubation and wash chambers serves to displace these proteins prior to reaching the elution chamber. There was a decrease in HRG concentrations across the wash chambers (from 132 to 70 nM, respectively). Similar trends were also observed in samples extracted from whole blood (from 505 to 26 nM, respectively). As previously noted, 150 mM imidazole served to elute HRG completely from the surface of the particles, but the binding and wash chambers contain slightly less imidazole (125 mM). During the wash steps, imidazole slowly displaces HRG from Ni^{II}NTA, which accounts for the general decrease in HRG concentration across the wash chambers. Greater than 90% of the total original concentration of HRG was removed from the final sample.

For nonspecific protein interfering agents (e.g., α-2-macroglobulin, HSA, lipoproteins), which may adsorb to the surface of the agarose particles, imidazole forms a salt barrier to prevent nonspecific carryover of these proteins. The oxygen transport protein, hemoglobin, is present in red blood cells at concentrations of 2–3 mM and represents an easily measured surrogate for nonspecific protein binding.[36] Processing of lateral flow immunochromatic malarial RDTs becomes a challenge in the presence of high background noise induced by the presence of hemoglobin and can lead to false readings. Analysis of the chambers for hemoglobin content showed that its concentration was dramatically reduced after the first wash chamber. The final eluate was virtually free of hemoglobin (Figure S-8, Supporting Information).

The final extracted samples were further analyzed by silver-stained SDS–PAGE gels and Bradford assay for total protein content. Silver-stained SDS–PAGE gels visually depict the contrast between the level of purification achieved in the elution chamber and the complexity of the original sample. After extraction, a very faint band is visible at approximately 67 kDa, the molecular mass of HSA (Figure 4). Finally, the total protein purification of the eluted sample was quantified and

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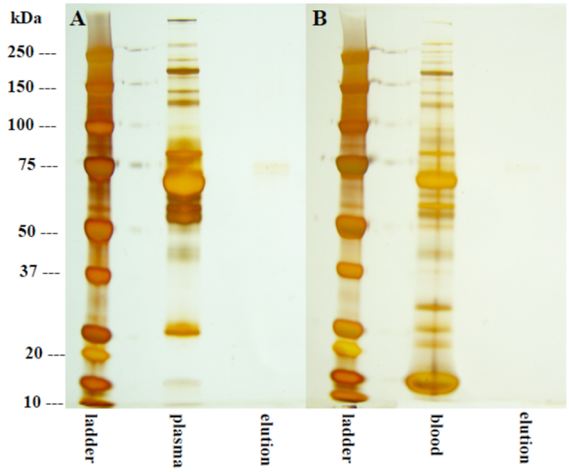

Figure 4. Silver-stained SDS–PAGE gel of the elution chambers of (A) plasma and (B) whole blood extractions (200 parasites/μL for both).

compared to the recovery of *pf*HRPII. Recovery of HRG from spiked plasma was reduced by 2 orders of magnitude, while a 1 order of magnitude reduction in HRG out of spiked blood was observed (Figure 5; Figure S-9, Supporting Information). Furthermore, the total amount of protein present in the final elution from either plasma or whole blood was 3–4 orders of magnitude less than that in the original sample. The recovery of targeted biomarker in the elution was 3–4 orders of magnitude greater than the amount of nonspecific contaminants.

Compared to the level of total protein purification obtained by the HisPur spin kit, purified target extracted from plasma using our cassette was 1 order of magnitude purer than the sample obtained using the spin kit (Figure 5; Figure S-9, Supporting Information). More strikingly, the spin kit appeared to copurify HRG with *pf*HRPII when extracting from whole blood, with recoveries of HRG at 37% versus 9% using the extraction cassette. Purified *pf*HRPII can be obtained by selective capture and purification of the biomarker from a complex matrix in under 30 min using only a hand-held magnet and processing solutions. This results in a final sample with a significantly improved ratio of target biomarker to nontarget protein interfering agents.

Concentration of *pf*HRPII for Improved Limits of Detection. The World Health Organization advocates that a threshold limit of detection for immunochromatic rapid diagnostic malaria tests be 200 parasites/μL; however, greater than 70% of WHO-qualified RDTs tested failed to give a positive reading at this limit.[5] The accessibility of these devices makes them attractive for malaria diagnosis at the point of care, but the reliability of the ensuing diagnosis limits their diagnostic utility. Furthermore, there is an increasing need to diagnose patients with asymptomatic levels of infection under 200 parasites/μL.[37] Though not exhibiting symptoms, this patient population can serve as parasite reservoirs for the continued spread of the disease. If the signal and limit of detection of these RDTs can be improved, a wider range of patients can be diagnosed with greater confidence.

The extraction cassette functioned to both isolate *pf*HRPII in high yields and simultaneously concentrate the biomarker 10-fold at clinically relevant concentrations of the parasite at and below the WHO limit of detection (Figure 6). In samples spiked to contain 12.5 parasites/μL, the yield of *pf*HRPII was

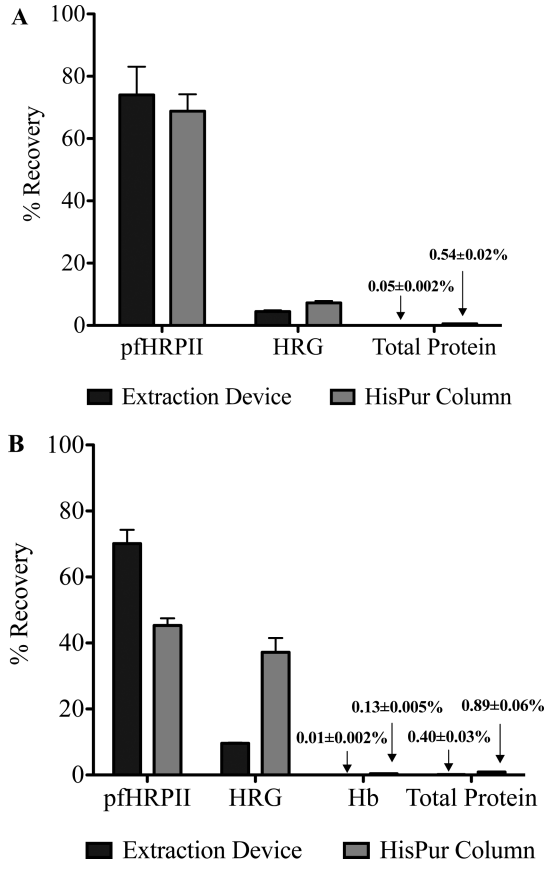

Figure 5. Comparison of recovery of nonspecific protein interfering agents and *pf*HRPII from (A) plasma and (B) whole blood using our extraction device and a commercially available HisPur Ni^{II}NTA spin column. HRG and total protein contents were quantified by ELISA and Bradford protein assay, respectively, for both matrixes. Very low levels of HRG, Hb (for blood only), and total protein were recovered from both matrixes (values given above where their respective columns would fall).

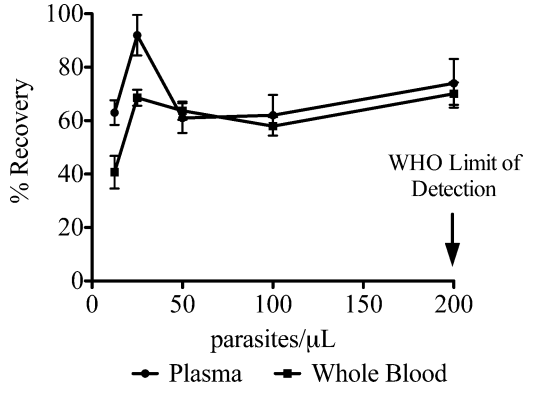

Figure 6. Recovery of *pf*HRPII from plasma (●) and whole blood (■) as a function of the parasite concentration (n = 6, mean ± SD). All recoveries were quantified by *pf*HRPII ELISA.

63.0 ± 4.6% from plasma and 40.7 ± 6.1% from blood. Furthermore, the release of purified biomarker into a 10 μL volume greatly enhanced the visual limit of detection of the commercially available SD BioLine malaria RDT (Figures S-10 and S-11, respectively, in the Supporting Information). Without processing through the cassette, no detectable signal was observed for any surrogate patient blood sample at or below

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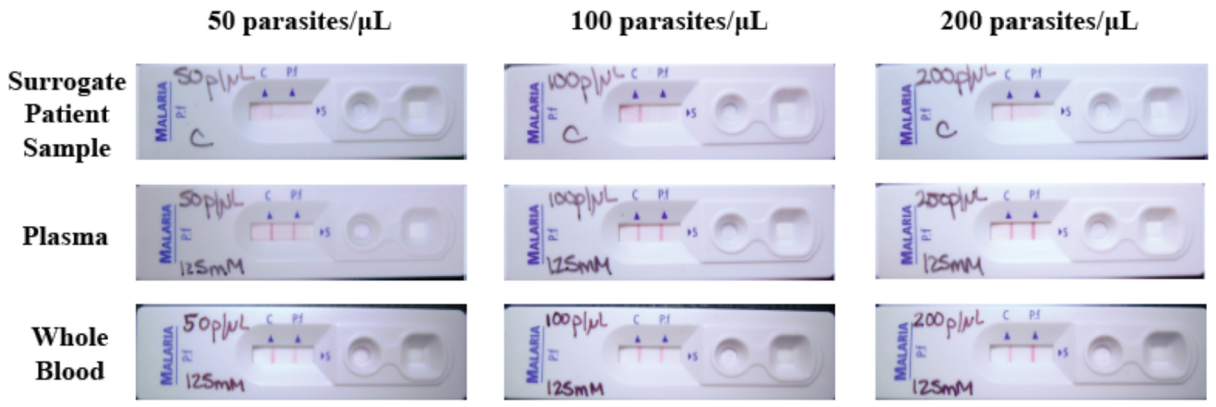

Figure 7. Improvement in the visual limit of detection of the SD BIOLINE malaria RDT after extraction from either plasma (middle) or whole blood (bottom) for 50 (left), 100 (middle), and 200 (right) parasites/μL as compared to mimic patient samples (top). The elution chamber from an extracted sample was spotted on the RDT and developed according to the manufacturer's specifications.

200 parasites/μL (Figure 7, top row). In contrast, for those samples first processed, positive test results were visualized reproducibly for samples with parasitemias as low as 25 parasites/μL (Figure 7, middle and bottom rows). The condition and integrity of the surrogate sample used for either direct application to the diagnostic test or extraction were identical. The future combination of this low-resource extraction cassette with readily available RDTs is a promising strategy for improving the diagnostic capabilities of these tests as well as extending into detection of clinically relevant parasitemias.

CONCLUSION

A low-resource, self-contained sample preparation device was created by incorporating a series of aqueous chambers separated by mineral oil valves into a single length of narrow-diameter tubing. This design extracts *pf*HRPII from human plasma and whole blood samples with >70% yield. In addition, processing of the sample results in a 1000-fold improvement in *pf*HRPII purification. The device concentrates the biomarker and enhances the performance of current RDTs. This simple sample preparation technology can be adapted for a wide range of sample preparation platforms and biomarkers given the ease of modularity in the device components. Future directions are aimed at improving the recovery and purity of biomarker by changing the bead and valve type as well as coupling the device to current patient sample collection techniques (e.g., dried blood spots).

ASSOCIATED CONTENT

Supporting Information

Additional information on experimental methods, gel images, extraction optimization figures, and pictures of RDTs pre- and postextraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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