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Magnetically-Enabled Biomarker Extraction and Delivery System: Towards Integrated ASSURED Diagnostic Tools

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Abstract

Diagnosis of asymptomatic malaria poses a great challenge to global disease elimination efforts. Healthcare infrastructure in rural settings cannot support existing state-of-the-art tools necessary to diagnose asymptomatic malaria infections. Instead, lateral flow immunoassays (LFAs) are widely used as a diagnostic tool in malaria endemic areas. While LFAs are simple and easy to use, they are unable to detect low levels of parasite infection. We have developed a field deployable Magnetically-enabled Biomarker Extraction And Delivery System (mBEADS) that significantly improves limits of detection for several commercially available LFAs. Integration of mBEADS with leading commercial *Plasmodium falciparum* malaria LFAs improves detection limits to encompass an estimated 95% of the disease reservoir. This user-centered mBEADS platform makes significant improvements to a previously cumbersome malaria biomarker enrichment strategy by improving reagent stability, decreasing the processing time 10-fold, and reducing the assay cost 10-fold. The resulting mBEADS process adds just three minutes and less than $0.25 to the total cost of a single LFA, thus balancing sensitivity and practicality to align with the World Health Organization's ASSURED criteria for point-of-care (POC) testing.

Graphical abstract

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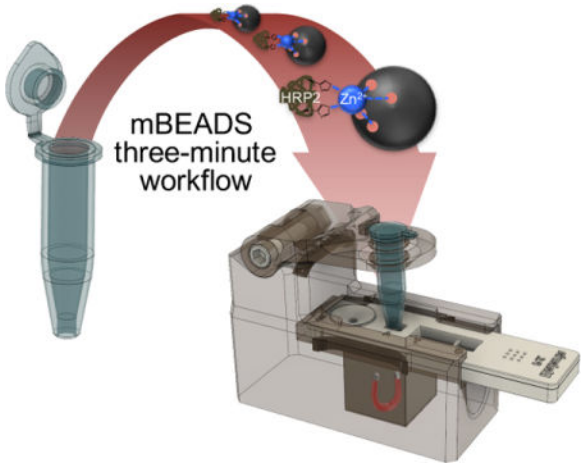

Keywords

ASSURED; Point-of-care (POC); lateral flow assays; rapid diagnostics tests; malaria; immobilized metal affinity chromatography (IMAC); biomarker enrichment

A. Introduction

Intensive control efforts have successfully reduced global malaria prevalence by 30% between 2000–2015.[1,2] Yet, populations in low and middle-income countries (LMICs) still bear a disproportionate burden of malaria disease due to limited diagnostic capacity and treatment availability. The gold standard for diagnosis of malaria parasites is blood smear microscopy, a 100+ year old technique that requires well-trained technicians and instrumentation not readily available in rural areas. These smear techniques are especially challenging for patients with low level disease, where the detection of parasites requires more extensive training than in high level infections. In recent years, the low cost and ease of use of lateral flow immunoassays (LFAs) has precipitated a paradigm shift away from microscopy.[1,3] The widespread use of LFAs has enabled the rapid identification and treatment of patients with high parasite burdens–an important contribution to successful malaria control efforts. However, LFAs lack sensitivity, which prevents the diagnosis of subjects with low levels of parasites who are often asymptomatic carriers of disease.[4–8] The failure of LFAs to identify these disease states leaves individuals untreated, thus maintaining a reservoir for continued transmission.[9–12] As global malaria efforts shift from disease control to disease elimination, existing LFA performance must be improved to accurately diagnose low levels of parasite at the point of care.

The World Health Organization (WHO) conducted an extensive analysis of commercially available LFAs for malaria diagnosis and concluded that the performance of many commercial malaria LFAs is sub-optimal at levels lower than 200 parasites μL^{-1}.[13] Diagnostic sensitivities in the single-digit parasites μL^{-1} are required to effectively identify the majority of asymptomatic individuals.[14] To address this LFA limitation, several new strategies have been developed that focus on enhancing the test line signal of the LFAs including: enzymatic-based signal enhancement,[15] silver-based enhancement,[16] dual gold

nanoparticle detection,[17] architecture-based enhancement,[18] and oligonucleotide-linked gold nanoparticle aggregation.[19] While the development of these new technologies promise potential solutions to this problem, commercialization and deployment of completely new tests will not only be time consuming and expensive, but will also require approvals at the national level and retraining of field healthcare workers. Alternatively, the integration of a biomarker enrichment procedure at the front end of the standard LFA workflow circumvents the need to redesign, remanufacture, and reapprove commercially available LFAs. Although several particle-based biomarker enrichment systems have emerged, most of them suffer from long assay times, need for a cold storage-chain, poor enrichment efficiencies, and most significantly, have not been shown to function in a whole blood matrix.[20–22]

Our laboratories have previously developed a sample preparation strategy utilizing magnetic-based metal chelation chemistry to enrich biomarkers from lysed whole blood. In these studies, nickel(II) nitrilotriacetic acid (Ni(II)NTA) magnetic beads were used to capture and extract histidine-rich protein 2 (HRP2) from lysed whole blood through an extraction cassette prior to LFA deposition.[23,24] Concentrating target biomarker from a large volume sample prior to LFA deposition was found to improve limits of detection, making it possible to measure parasite levels lower than the 200 parasites μL^{-1} WHO standard.

More recently, we reported that biomarker deposition onto an LFA within a housing device enabled direct transfer of HRP2 onto a selected commercially available LFA.[25] This discovery removed the need for an additional extraction cassette and reduced the user steps needed to complete the procedure. However, the system still suffered from long incubation times, high cost per assay, a cumbersome user interface designed for only one commercial LFA, and the need for laboratory equipment. Here, we report the development of an integrated mBEADS device and workflow that overcomes these failure points to meet the WHO's ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable) criteria for field deployment.[1,26,27] This report focuses on using the ASSURED criteria as a framework to systematically evaluate all assay components and advance our proof-of-concept technology to a POC system.[28]

We address affordability and rapid biomarker capture by optimizing capture beads solid phase and chemistry. The optimal capture bead and all assay components are incorporated into a robust, single-use sample preparation tube that when combined with our battery-powered handheld mixer provides a deliverable assay platform. The 3D-printed device is designed to be user-friendly and to interface with five common LFAs that are used in low-resource areas for specific detection of the target biomarker. Although this system requires some equipment, the battery-powered mixer, mBEADS device, and commercial LFA are compact and can be used on site, which is in line with the equipment-free principle put in place by the WHO.[28] The integrated mBEADS workflow increases the sensitivity of the leading LFAs to be able to detect antigen concentrations in blood associated with single digit parasitemias, while taking less than three minutes to complete, and contributing less than $0.25 to the total assay cost. This sample preparation system is well-aligned with the ASSURED criteria developed by the WHO for POC diagnostics.

B. Experimental Methods

B.1 Sample Preparation

All reagents and buffer components were purchased through Fisher Scientific unless otherwise noted. Pooled whole human blood with a citrate phosphate dextrose anticoagulant was used in each experiment (Bioreclamation IVT, USA, Item: HMWBCPD). *Plasmodium falciparum* parasite (D6 strain) was cultured in our lab as a source of HRP2 for all experiments.

B.2 Packed Bead Volume as a Standard for Bead Capture Studies

Stock magnetic beads provided by each manufacturer varied by concentration, size, polydispersity, surface functionalization, and cross-linked solid phase density (Table S1). This made direct comparisons between the different solid phases challenging. To compare bead performance, bead volumes were standardized by loading 10 μL of homogenously mixed stock beads into a 1 mm diameter 25 μL microdispenser capillary tube (Drummond, USA) that was then closed with tube sealing compound (Mooremedical, USA). Beads were than magnetically compressed to the bottom of the tube using a 1″ Neodymium magnet (K&J Magnetics, USA) and tapping the tube against the magnet for 2 min. A caliper was then used to measure the height of the magnetically packed beads inside the capillary tube. This measurement was performed in triplicate for all beads evaluated. Based on the diameter of the tube and the packed magnetic bead height, the packed bead volume was determined and normalized to 5.5 mm^3. Packed bead volume of 5.5 mm^3 corresponds to the following volumes of stock bead solutions: 10 μL of Qiagen, 2.1 μL of Cube Biotech, 5.7 μL of Bioneer, and 9.5 μL of CloneTech beads. See supplemental information for more detail (Figure S1, Table S2).

B.3 Bead Capture and Binding Capacity Studies

Four immobilized metal affinity chromatography (IMAC) magnetic beads were screened in these studies: Cube Biotech PureCube Ni(II)NTA MagBeads (Germany), Qiagen Ni(II)NTA Magnetic Agarose Beads (Germany), Bioneer AccuNanoBead Ni(II)NTA (Korea), and CloneTech His60 Ni Magnetic Beads (USA). To evaluate bead performance, whole blood was lysed by mixing 1:1 with lysis buffer (100 mM phosphate buffer pH 8.0, 600 mM NaCl, 20 mM Imidazole, and 2% Triton X-100 solution). *P. falciparum* D6 parasite culture preparations were spiked into lysed whole blood to achieve concentrations ranging from 65 – 4000 parasites μL^{-1}. The standardized bead volumes were added to 100 μL of lysed whole blood parasite preparations in a 96 well plate (Corning, USA) and allowed to incubate for 10 min on a plate shaker (VWR, USA). After incubation, magnetic beads were separated by a 96-well plate magnet (MagWell, USA), supernatants were collected, and an enzyme-linked immunosorbent assay (ELISA)[23] was performed to measure the residual HRP2 not captured by the beads. Bead capture efficiency percentage was calculated based on these HRP2 concentrations.

B.4 IMAC Divalent Metal Screening

Agarose beads functionalized with nitrilotriacetic acid (NTA) chelating ligand loaded with one of four divalent metals (Co^{2+}, Ni^{2+}, Zn^{2+}, and Cu^{2+}) were procured from Cube Biotech. Per manufacturer datasheet, the metal ion capacity of these beads is approximately 12 μeqv M^{2+}/mL resin. Binding kinetics were evaluated for each bead type by examining the concentration of HRP2 left in sample supernatants as a function of incubation time. Standardized volumes of magnetic beads were added to 100 μL lysed whole blood spiked with *P. falciparum* D6 parasite culture to a final concentration of 200 parasites μL^{-1}. At 1, 2, 5, and 10 min incubation times, beads were separated using a 96-well plate magnet and supernatants were collected. ELISA was used to estimate residual HRP2 not captured by the beads.[23]

B.5 Single-Use Bead Lyophilization Compatibility and Stability Assessment

Ten different assay compositions (100 μL) were lyophilized in 0.5 mL Flat-Cap PCR Tubes (Fisher Scientific, USA) to assess optimal lyophilization parameters. Trehalose and polyethylene glycol (PEG) 8000 were supplied by Sigma-Aldrich (USA) and evaluated as potential additives during the lyophilization process to maintain the integrity of the three dimensional agarose network.[29] Samples were frozen at −80 °C, placed in the lypohilizer (Labconco, USA), and dried at −80 °C under vacuum (0.133 mBar). After lyophilization, samples were stored in a plastic bag equipped with silica desiccants. Fifty microliters of hydrating lysis solution (2% triton X-100 in D.I. H_2O) was added to lyophilized samples. Samples were rehydrated and homogenized by mixing for 5 sec on a battery-powered orbital mixer made from a personal massager (Wahl Corporation, prod. no. 4293).[30] Sample preparation tubes were removed from the orbital mixer for the addition of 50 μL of 200 parasites μL^{-1} blood, then returned to the mixer for a 1 min incubation. Samples were then placed on a magnetic rack (Life Technology, USA) to separate the beads from the sample. Each sample supernatant was analyzed by HRP2 ELISA to determine capture efficiency.[23] To confirm sample tube stability, this experimental protocol was performed with sample tubes that were housed in a plastic bag containing silicon desiccant packets for 386 days after lyophilization.

B.6 Device Design and Development

The mBEADS device utilizes magnetic bead enrichment to deliver concentrated HRP2 onto commercially available malaria LFAs to enhance test performance. This was achieved through the use of 1) flexible plastic spacers constructed from zip ties (McMaster-Carr, USA) in the inner rails of the LFA slot to center the alignment of each LFA, 2) a multi-level backstop to position the sample deposition pad of each LFA directly below the sample tube holder, and 3) a soft rubber stopper to enable the sample tube to be depressed for deposition pad contact (Figure 1). The cantilever-based sample tube holder is a feature that simplifies sample handling analogous to a common office stapler: it flips open to accommodate loading the sample deposition tube, flips closed for magnetic bead collection, and depresses for bead delivery onto the LFA sample pad. This design also incorporates a lock to secure the sample deposition tube, minimizing sample loss and positioning variability when the top is flipped closed. The final mBEADS device reported here underwent twenty-nine iterations

to improve usercentric features, make compatible with 5 commercial RDTs, and optimize bead deposition (Figure S2). The mBEADS device was fabricated using 3D printing technology allowing for potential modifications to be readily made to accommodate alternate LFAs if desired. Utilizing this 3D technology provides substantial flexibility compared to alternative diagnostic enhancements.[31]

The bead transfer devices were designed using Fusion 360 software (Autodesk). Since manufacturers adopt unique form factors for their tests, mBEADS was engineered to accommodate five commercially available malaria LFAs for *Plasmodium falciparum* HRP2: Paracheck (Orchid Biomedical Systems), SD Bioline (Standard Diagnostics Inc), First Response (Premier Medical Corporation Ltd.), Care Start (AccessBio), and ICT Pf (ICT International) (Figure 1C). The devices were 3D printed (ProJet 3510 HD *Plus*, 3D Systems, USA) using VisiJet M3 Crystal material (part no. 2184–905).

B.7 Magnetically-assisted Bead Gathering Study

To standardize the end-user workflow, the time required for beads to be gathered in the presence of a magnetic field was measured using time-lapse photography. Magnetic beads (5.5 mm^3 μL, Cube Biotech) were added to microcentrifuge tubes with 50, 100, 200, and 300 μL of whole blood lysed with 1:1 v/v, blood/lysis buffer, to a total volume of 100, 200, 400, and 600 μL, respectively. The total lysed blood volumes were vortexed briefly to achieve a homogenous spatial distribution, and then placed in a holder 5 mm above a 1″ Neodymium magnet, replicating the magnetic field configuration when the sample deposition tube is placed in the mBEADS device, in its closed position. Additional lighting was used to create a clear distinction between the pixel intensity of the settled beads and that of lysed blood. A video camera (Edgertronic, USA) was set to capture 10 frames per second for 90 s. Images were loaded into custom image-processing software, written in MATLAB v8.5 (MathWorks, USA), and binary image segmentation isolated the clustered beads at the bottom of the tube from the rest of the blood, based on pixel intensity. The combination of lysis buffer and intense illumination resulted in a clear distinction between the beads and lysed blood solution. A connected component algorithm was used to quantify the area (in $pixels^2$) of the settled bead cluster in each frame.

B.8 Parasite Titration for Limit of Detection Determination

Enhanced test samples were prepared by hydrating the lyophilized samples with 50 μL of lysis solution as described above. The optimized 1 min mixing time determined in the incubation studies was used for the lyophilized Zn(II)NTA single-use sample tubes. Samples were prepared with 1, 2, 5, 10, 25, 50, 100, 150, and 200 parasites μL^{-1} for both unenhanced controls and mBEADS enhanced LFAs using each of the following tests: Paracheck, First Response, and ICT Pf. Fifty microliters of blood, spiked at the appropriate parasitemia, was then added to the sample tube and the sample was placed back on the handheld orbital mixer for 1 min. The sample deposition tube, a modified PCR tube, was inserted into the deposition device in the ‘open’ position. PCR tubes were modified by cutting off the closed end with a razor blade using a standardized custom tool to provide a consistent aperture large enough to facilitate efficient bead transfer upon contact with the LFA conjugate pad (~2 mm diameter). The cap of each PCR tube was sealed with Teflon tape to prevent

magnetic bead loss in the crevasses of the lid. After 1 min of mixing, samples were removed via pipette and transferred to the sample deposition tube. The LFA was inserted into the mBEADS device and the device top was flipped to position the sample deposition tube directly over the conjugate pad (aligned with the center of the 1″ magnet). The depositions tube's millimeter-diameter tube aperture creates a stable surface tension valve that retained the sample and allowed beads to collect at the meniscus formed at the bottom of the tube during the 1 min bead collection.[32] The developed surface tension valve prevents premature transfer of magnetic beads to the LFA during magnetic bead gathering step. The cantilever-based tube holder was then depressed briefly by the user in order for the bead-filled meniscus to make contact with the conjugate pad, breaking the surface tension valve, and transferring the beads to the LFA. The LFA was removed from the device and running buffer modified with 500 mM imidazole was added to the LFA running buffer reservoir. For unenhanced controls, 5 μL whole blood samples were pipetted onto the conjugate pad and LFAs were used per manufacturer's instructions.

The HRP2 test line signals generated were quantified with an ESEQuant Lateral Flow Reader (Qiagen Inc, USA) between 25 and 30 min after sample deposition. Wicking pads were removed and test strips were inserted into the reader to measure reflectance (test line colorimetric intensity) of the control and test lines for all samples. Test line intensities were analyzed in the ESE Quant LF studio software with a peak area at a fixed baseline using a signal threshold of 30 mV. Experiments were performed in triplicate and reported as average peak area.

B.9 Determining the Limit of Detections for LFAs

Each sample, at varying concentrations, was deposited onto an LFA in triplicate, and the mean test line peak areas were calculated

$$X_{LOD} = \frac{3(\text{error in } \gamma - \text{intercept})}{\text{slope}} \quad (1)$$

to generate one data point for each concentration. For full parasite titration experiments, linear regression was used to develop a statistical model to estimate concentration as a function of signal intensity. The error in the γ-intercept and the slope of the regression line were inserted into the following equation to provide the limit of detection (LOD) (Equation 1). Linear regression and limit of detection calculations were performed for both enhanced and unenhanced LFAs.

B.10 Efficiency of HRP2 Deposition by ELISA

HRP2 ELISA was used to quantify residual HRP2 in specimens after incubation with magnetic HRP2 capture beads and subsequent deposition onto the LFA.[23] After deposition of the beads onto the LFA using the mBEADS device, the remaining sample solution and residual beads were collected in the sample preparation tube via centrifugation. Residual beads were separated using a magnetic rack and supernatants were removed and analyzed using HRP2 ELISA. These beads were magnetically separated, washed three times with

wash buffer (1× PBS, 0.1% Tween-20), and incubated in elution buffer (50 mM phosphate buffer, 300 nM NaCl, 500 mM imidazole buffer, 0.1% Tween-20) for 5 min. The eluent was then examined by ELISA to quantify the captured HRP2 left behind on beads.

B.11 Increased Sample Volume Study

To determine the improvement in sensitivity that mBEADS provides with larger sample volumes, additional reagents were prepared at larger total volumes. Lyophilized reagents were scaled appropriately for the desired blood sample volume. The same optimal lyophilization parameters described above were used, except the lyophilization buffer (50 mM phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% trehalose) mirrored the total sample volume to be added. For example, single-use sample tubes prepared for the 400 μL total sample volumes were lyophilized with 400 μL of lyophilization buffer. All samples were spiked to 1 parasite μL^{-1} for the 50, 100, 200, and 300 μL blood sample volumes used (reported in results as total volumes after the 1:1 lysis method). Mixing times and bead settling times varied: 1 min mixing and 1 min settling time for 100 and 200 μL, and 2 min mixing and 2 min settling time for 400 and 600 μL. Using the mBEADS system, each sample was deposited onto a Paracheck LFA and read, following the suggested 30 min development time, using an ESEQuant Lateral Flow Reader. For control samples, 5 μL whole blood samples were pipetted onto the conjugate pad and developed per manufacturer's instructions. A F-test was performed on the data series to determine if the variances observed in the data set were significant.

C. Results

Motivated by the lack of field deployable LFA enhancement technologies, mBEADS was developed to better align a proof-of-concept biomarker enrichment strategy with the WHO's ASSURED criteria. The mBEADS approach has a straightforward workflow composed of a single-use patient sample tube, a transfer pipette, a portable battery-powered mixer,[30] and a user-friendly 3D-printed LFA alignment and bead deposition device that increases the performance of LFAs currently used in the field. Together, these components overcome prohibitive barriers in previous technologies enabling the translation of this sample preparation technique to point of care for countries that are embarking on malaria elimination campaigns.[33] Simply by collecting more whole blood sample from the patient, the proposed system employs rapid magnetic bead assisted capture, concentration, and delivery of target biomarkers to LFAs to achieve the increased diagnostic sensitivity needed to be effective in elimination campaigns.

C.1 Magnetic Capture Bead Selection

To make a biomarker enrichment system that is field deployable, improvements in processing time, reagent stability, and assay cost had to be made to our previous approach. The magnetic capture bead, the chemically functional component in this assay, was targeted for system optimization to address these limitations. The system was optimized by exploring the interactions between the capture bead and HRP2. The capture beads used in this study are immobilized metal affinity chromatography (IMAC) magnetic beads that exploit the affinity of the high number of intrinsic histidines in HRP2 towards divalent metals

immobilized on a magnetic solid support. This is an ideal capture bead to use in low-resource settings because it is robust, lacks biological capture agents, and provides a gentle elution strategy that is compatible with LFAs. The two functional components of these magnetic beads are the solid phase and the immobilized divalent metal. Previous studies that used IMAC chemistry to isolate HRP2 employed 20 μL of Ni(II)NTA Qiagen magnetic beads, calculated to be 11.0 mm^3 of magnetically packed beads, captured approximately 80% of HRP2 available in a 100 μL lysed whole blood sample spiked to 200 parasites μL^{-1}. This capture was achieved after a vigorous 10 min mixing step.[25]

Until now, no studies have been performed that investigate how solid phase and choice of immobilized coordinating metal ion impact HRP2 capture. To evaluate the impact of magnetic bead solid phase on HRP2 capture, four commercially available IMAC solid phases charged with divalent nickel were procured and tested for HRP2 capture performance. HRP2 capture efficiencies were compared across manufacturers using a standardized magnetic packed bead volume of 5.5 mm^3 (Figure S1) with a 10 min mixing step (Figure 2A). Cube Biotech beads provided the most effective solid phase, capturing 80 – 85% of total HRP2 available in samples ranging between 65 – 4000 parasites μL^{-1}. Additionally, the Cube Biotech solid phase showed no significant decrease in percentage of HRP2 capture across the wide range of parasite densities tested. It was concluded that HRP2 binding saturation was not reached and 5.5 mm^3 packed bead volume was a sufficient bead volume to be used in further studies. Additionally, Cube Biotech beads were the most cost efficient option, which is examined further in the discussion.

Rapid time to result is crucial in POC assays, and the previous Ni(II)NTA Qiagen bead sample preparation workflow suffered from long mixing times for HRP2 capture.[34] Although an improved solid phase for HRP2 capture was identified, a 10 min mixing step was not sufficient to capture all the available HRP2. In IMAC chemistry, metals that are strongly fixed to a solid support mediate the interaction between the solid phase and the target. The most common method uses a solid phase equipped with the NTA ligands to coordinate Ni^{2+} with four valencies (Ni(II)NTA) to fix it in place. The remaining two valencies on Ni^{2+} are then available to coordinate to histidine rich moieties, such as HRP2 or the archetypal “His-tag” used in protein purification.[35] However, reports suggest that Zn^{2+} has a uniquely high binding affinity to HRP2 compared to other divalent metals.[36,37] To improve binding efficiency and better understand how immobilized divalent metals kinetically interact with HRP2, the relationship between divalent metal species and mixing time was investigated. HRP2 capture was evaluated as a function of time using Cube Biotech beads equipped with Ni^{2+}, Zn^{2+}, Co^{2+}, or Cu^{2+} (Figure 2B). Zn(II)NTA, Cu(II)NTA, Co(NTA), and Ni(II)NTA surface functionalization was found to capture 99.7 ± 2.7%, 99.4 ± 6.8%, 95.7 ± 2.1%, and 85.6 ± 5.3% of the available HRP2 employing a 10 min mixing step, respectively. However, at shorter mixing times, which are needed for POC diagnostic systems, the surface functionalization was found to have a greater impact. Zn(II)NTA functionalization enabled the capture of 98.2 ± 2.7% of the available HRP2 with a short 1 min mixing step, while Cu(II)NTA, Co(NTA), and Ni(II)NTA surface functionalization was found to capture 8.7 ± 6.4%, 59.9 ± 1.9%, and 70.1 ± 10.4 of the available HRP2 with a 1 min mixing step, respectively.

Thus, the identification of Cube Biotech, an improved magnetic bead solid phase, and optimal Zn(II)NTA surface functionalization allowed for a 10-fold reduction in mixing time, while also increasing HRP2 capture by 20% compared to our previous method.

C.2 Development of a Stable Single-Use Sample Tube

A novel single-use patient sample tube containing the necessary biomarker capture components was developed to minimize user steps, increase reagent stability, and reduce variability in assay performance. Lyophilization of agarose beads, with the addition of excipient lyoprotectants such as sugar and hydrocarbons, has previously been reported to preserve the 3D structure and chromatographic properties of agarose beads.[29] To investigate the impact of the inclusion of all assay components in a single-use sample tube, a lyophilization composition screen was performed. In these experiments several combinations of PEG 8000, trehalose and other stabilizing agents, as well as the mandatory assay reagents including: 5.5 mm^3 Cube Biotech Zn(II)NTA beads, sodium chloride, blocking imidazole, and phosphate buffer pH 8.0 were lyophilized in single-use tubes and evaluated for HRP2 capture efficiency after mixing on the battery-powered mixer for 1 min (Figure 3A). The best performing lyophilization composition consisted of 50 mM phosphate buffer pH 8.0, 300 mM sodium chloride (IMAC Buffer), 10 mM blocking imidazole, and 10% trehalose (Figure 3A.6). Single-use sample tubes prepared with this composition captured ~100% of the HRP2 present in a 50 μL whole blood sample (100 μL total sample volume after addition of hydrating lysis solution).

Long-term stability of the lyophilized single-use tube was also analyzed. Enclosed in a plastic bag containing desiccating silicon packets at room temperature, these single-use tubes remained stable and maintained the ability to capture 100% of HRP2 present in a 50 μL whole blood sample up to 386 days after their preparation (Figure 3B). No bacterial growth was observed in the hydrating lysis solution during the same timeframe, suggesting that all sample processing reagents have a shelf life of over 1 year at room temperature maintaining the stability requirements of the WHO ASSURED criteria.[28] The development of this single-use sample tube and the employment of our battery-powered mixer provides a deliverable high-throughput assay with minimal consumables.

C.3 Magnetically-assisted Bead Gathering to Deliver the Greatest Number of Biomarkers to LFAs

The results from this study defined the final steps in the mBEADS workflow using 50 μL whole blood samples (100 μL total sample volume). The final steps in the mBEADS workflow involve operating the user-centric device to align the LFA, use the on-board magnet to gather the magnetic beads, and deliver them into the LFA sample port employing the cantilever deposition action. Following the rapid 1 min capture of HRP2, all components of the stable single-use tube are transferred to a sample deposition tube on the mBEADS device for a magnetic-assisted gathering step. To promote the best delivery of the magnetic beads onto the LFA, which now harbor all of the HRP2 from the sample, it is critical that the greatest volume of beads be gathered at the bottom of the deposition tube. To that end, magnetic-assisted bead gathering time was investigated to provide the minimum time necessary to gather the maximum number of magnetic beads prior to deposition.

Time-lapse photography was used to determine the time necessary for magnetic beads to gather at the bottom of the sample tube. The magnetically gathered bead packet that forms at the bottom of tube, denoted as 2D bead settled cluster area, was monitored and normalized to the final settled cluster area (Figure 4). For each volume tested, the transient bead collection was highly reproducible (i.e. the settled cluster area increases in a similar manner for each replicate within a given volume). According to a Students t-test, there was no statistically significant difference between the time that it took for 90% of the beads to settle for lysed whole blood sample volumes of 100 and 200 μL (4.7 +/− 1.6 s and 4.2 +/− 0.67 s, respectively). However, there was a statistically significant difference in the bead settling time for 400 μL (13.2 +/− 2.9 s) and 600 μL sample volumes (28.6 +/− 5.5 s, $p<0.05$) (Figure 4C). Consequently, a 1 min bead gathering step was adopted for volumes of 100–200 μL and a conservative 2 min bead gathering time for volumes of 200–600 μL.

C.4 m BEADS Biomarker Delivery Efficiency and Enhancement of Leading Commercial LFAs

The most important operation of the mBEADS system is to escort as many possible HRP2 biomolecules from a blood sample to a LFA to increase the test line intensity. Therefore, after the components and workflow of mBEADS were defined, biomarker to LFA delivery efficiency was evaluated. An illustration of the full mBEADS process is provided for reference (Figure 5). By subtracting the residual HRP2 left in the sample supernatant (uncaptured HRP2) and on the beads remaining in the sample tube after deposition (captured but not deposited) from the total amount of HRP2 in the sample, a biomarker delivery efficiency was determined (Table S3). The optimized device employing Zn(II)NTA single-use tubes was able to deliver approximately 75% of the total HRP2 protein in the sample onto the LFA.

To determine the increased sensitivity afforded by mBEADS, LOD calculations were performed on data sets composed of standard and mBEADS enhanced LFAs using mock clinical samples over parasite densities ranging from 1 to 200 parasites μL^{-1} for Zn(II)NTA single-use tubes. These studies were performed for each of the following LFA types: Paracheck, ICT Pf, and First Response.

The other two test types that the mBEADs device was designed to incorporate were excluded due to trouble procuring a sufficient number of tests from the manufacturers. Dose-response curves used to calculate the limit of detection for two of the three available malaria LFAs are shown in Figure 6. The greatest enhancement was seen with First Response tests, where the mBEADS process yielded a LOD at 1.3 ± 0.1 parasites μL^{-1}. Table 1 lists the LODs for each LFA with and without mBEADS enhancement. Detection limits are also reported in the corresponding quantities of HRPII because the mock clinical samples were generated from a high parasite density stock culture.[38]

The practical whole blood volume that can be readily acquired employing lancets that are supplied in the commercially available LFA kits used in this study is 50 μL. Thus, the experimental protocol to determine the sensitivity of mBEADS LFAs used 50 μL whole blood samples. However, the approach proposed can be easily adapted to incorporate larger patient blood volumes by manipulating the reagent volumes in the single-use sample

preparation tubes and incrementally increasing mixing and bead settling times. Using improved lancet technology, finger prick blood collections can acquire up to 300 μL of patient whole blood, further increasing the total amount of HRP2 that can be captured (Figure S4) and delivered to LFAs. The impact of processing larger whole blood volumes on the HRP2 test line development was evaluated employing mBEADS (Figure 7). Processing larger whole blood volumes increased LFA test-line signal intensity at single parasite densities suggesting that the collection and processing of whole blood volumes larger than the 50 μL used in this study could lead to an even more thorough detection of the asymptomatic infectious reservoir.

D. Discussion

Intensive control efforts have significantly reduced malaria disease burden, and many regions are undergoing a crucial shift from malaria control to malaria elimination.[3–12,40–42] High sensitivity detection at the point of care is an essential diagnostic component as malaria elimination campaigns scale up globally. One of the greatest challenges facing elimination campaigns is the effective identification of individuals with asymptomatic disease who may serve as a parasite reservoir.[43,44] Using the WHO's ASSURED criteria as a framework, we have developed a biomarker enrichment approach that integrates simple, user-centric sample preparation with commercially available LFAs to generate very high sensitivity measurements of *Plasmodium falciparum* HRP2. A link to a training video and a detailed protocol showing the entire process can be found in the supplemental information (Video S1 and supplemental methods). Using mathematical models, Slater *et al.* showed that diagnostic sensitivity of 200 parasites μL^{-1} at the point of care allows for the detection of only 55% of the infected population.[14] In this study, some of the leading *Pf* malaria LFAs were selected to be integrated with mBEADS. The LODs for these LFAs range from 21–50 parasites μL^{-1}. The improvement in the detection limit of LFAs to single-digit parasitemia increases the detection of the infectious reservoir to 95%.[14] Thus, the greatest advantage of the integration of mBEADS with existing LFA technology is achieving the single parasite density detection limits that will be necessary for malaria elimination campaigns without the need to remanufacture commercial tests. Additionally, while some current lab-based assays such as PCR or ELISA can approach this level of sensitivity, unlike LFAs, they cannot be deployed at scale or in low resource settings where elimination campaigns will operate.[45]

The progression from our proof-of-concept technology to this field-deployable mBEADS platform was guided by selection criteria that sought to improve commercially available LFA sensitivity without adding a cumbersome process, burdensome cost, or lengthy time requirements. We believe that by further refinement we can reduce this two-tubed system to a one-tubed system to further reduce user step. Additional cost per assay is rarely addressed in the development of LFA enhancement strategies but is one of the greatest prohibitive barriers to their translation and acceptance in low and middle-income countries. The most expensive component of particle based enhancement systems is often the biomarker capture bead. In our previous work, the chosen bead system was ~$2.20 per assay.[25] To better visualize the relationship between HRP2 capture and bead cost, percent HRP2 capture efficiencies at 200 parasites μL^{-1} were plotted against the bead cost per assay for each bead manufacturer (Figure 8). The most desirable beads are those that are inexpensive beads and

most effectively capture HRP2, which are found in the top left portion of the graph. The Cube Biotech Zn(II)NTA magnetic beads selected for mBEADS were not only the most effective HRP2 capture magnetic bead, capturing ~100% of HRP2 with a 1 min mixing step, but also the least expensive at $0.09 per assay. The total additional cost of employing the complete mBEADS process is $0.25 per LFA, permitting the mBEADS device has a lifetime of 500 uses.

Moreover, of the particle-based sample preparation systems reported in the literature,[20–22] this is the only one that has been demonstrated to function in whole blood, purifying HRP2 from components of blood that may interfere with LFAs.[20–22] The magnetic bead deposition was tailored to have minimal sample carryover volume, resulting in improved test clearance. Furthermore, it likely prevents the transfer of LFA interferents, such as autoantibodies associated with human rheumatoid factor, and human anti-mouse antibodies that induce false positive test results.[46] The deposition of a small number of beads also provides a theoretical maximum HRP2 binding and delivery capacity that would provide tolerance against the hook effect phenomenon known to give false negative results.[47]. To this end, the general performance and robustness of widely-deployed LFAs is increased by employing mBEADS.

A. Conclusion

Malaria eradication has become a promising and realistic target due to global initiatives to diagnose infection, treat positive cases, and decrease transmission rates. However, current POC diagnostics are not sensitive enough to detect low parasite densities present in some asymptomatic patients. For malaria elimination campaigns to be effective, POC diagnostics must be able to detect the single digit parasitemias that are associated with this asymptomatic patient reservoir.[14] Instead of dealing with the challenges of developing, implementing, and gaining governmental approval of a new next-generation rapid diagnostic tests to achieve this, we propose the enhancement of current, widely used technologies using the integrated mBEADS system. The ASSURED-criteria aligned mBEADS system enhances the preeminent deployable LFAs to achieve the diagnostic sensitivity needed for elimination campaigns to finally eradicate malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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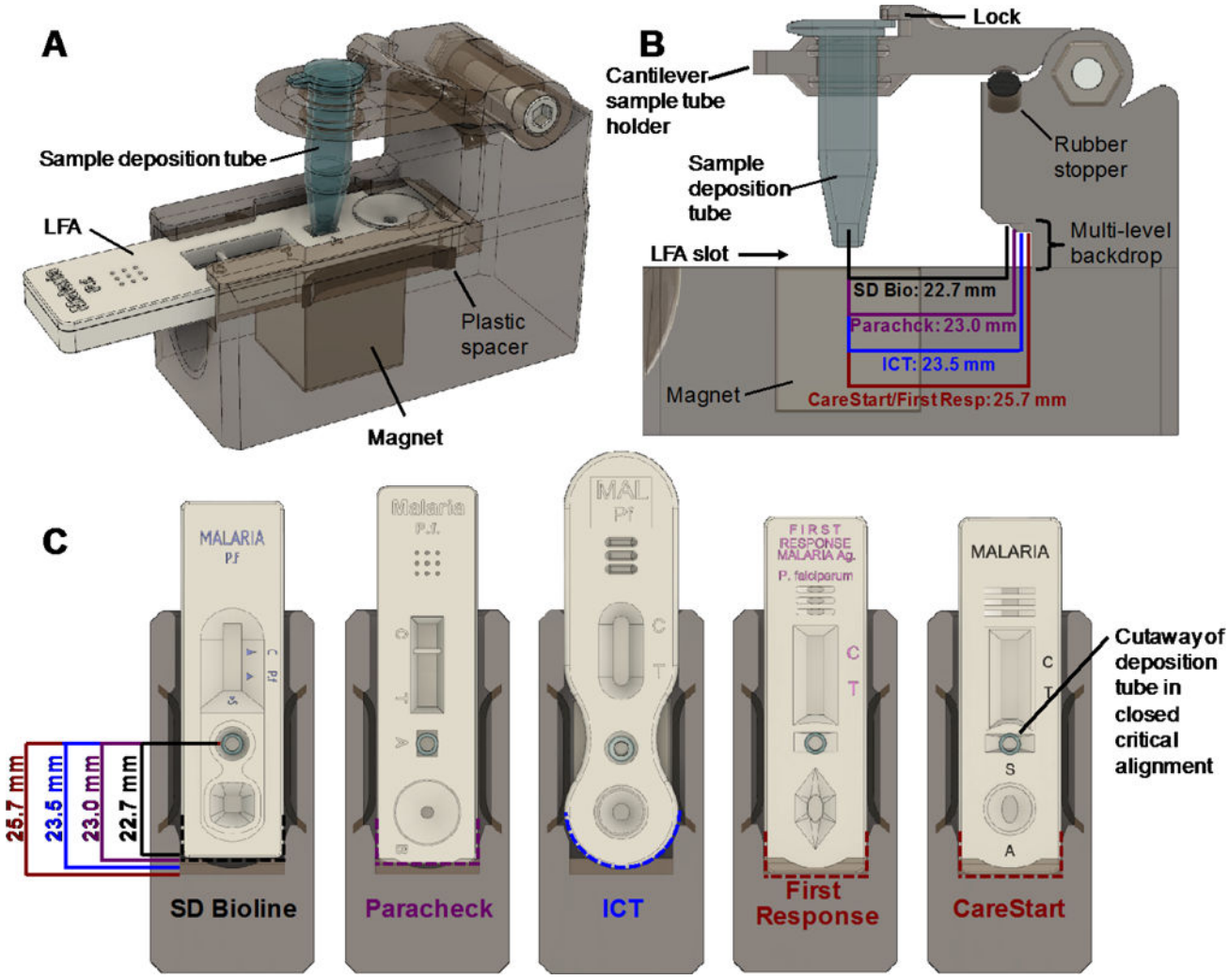

Figure 1.

3D device design for automatic LFA alignment and magnetic bead transfer. A) 3D rendering of the mBEADS transfer device shown with Paracheck LFA and sample deposition tube inserted. B) Cross-sectional side view of the bead transfer device depicting the multi-level backstop for aligning each LFA despite the various distances from the end of the LFA to the center of the sample deposition port. C) Cross-sectional top view depicting the variation in depth for each LFA after insertion into the bead transfer device. Dotted lines indicate the backstop position for each LFA.

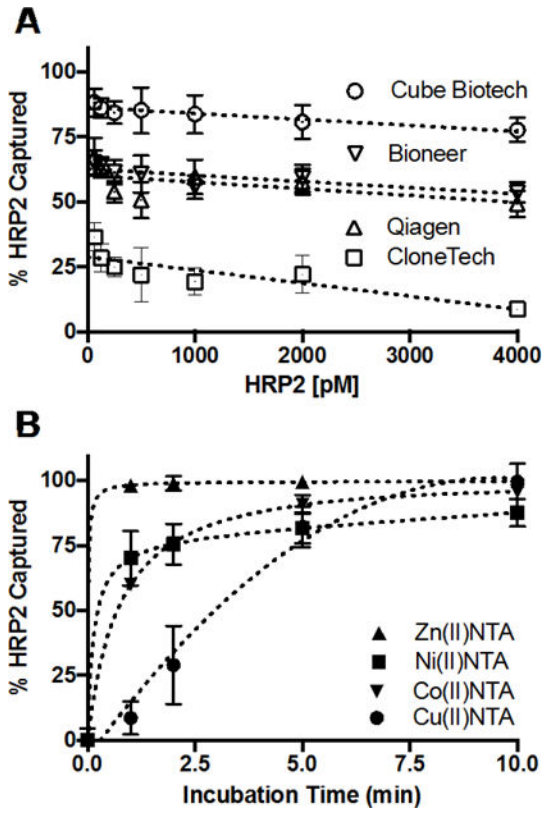

Figure 2.

Bead solid phase and divalent metal selection. A) The HRP2 capture performance of four different commercially available Ni(II)NTA IMAC solid phases over a range of parasite densities employing a 10 min incubation time. B) Cube Biotech beads equipped with four different divalent metals capture HRP2 in 200 parasites μL^{-1} samples as a function of time. Both graphs were fit with nonlinear best-fit trend lines

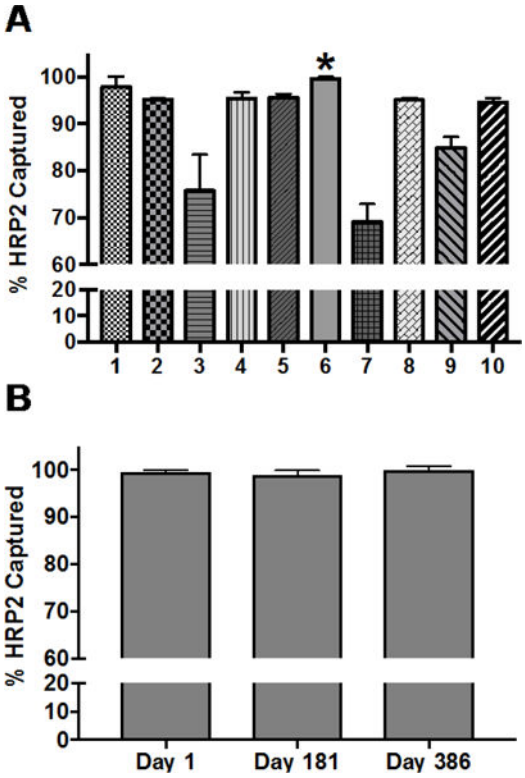

Figure 3.

Lyophilization composition studies. A) The capture of HRP2 by 5.5 mm^3 of Cube Biotech Zn(II)NTA beads in a whole blood sample, after lyophilization in ten different lyophilization compositions: 1) IMAC buffer, 10% PEG 8000 10% trehalose 2) IMAC buffer, 10% trehalose 3) IMAC buffer, 10% PEG 8000 4) IMAC buffer 5) IMAC buffer, 10 mM imidazole, 10% PEG 8000, 10% trehalose 6) IMAC buffer, 10 mM imidazole, 10% trehalose*7) IMAC buffer, 10 mM imidazole, 10% PEG 8000 8) IMAC buffer, 10 mM imidazole 9) lyophilized beads alone 10) non-lyophilized beads. * Lyophilization composition used in further testing. B) Single-use patient sample tubes were evaluated for HRP2 capture efficiency on Day 1, Day 181, and Day 386 after lyophilization.

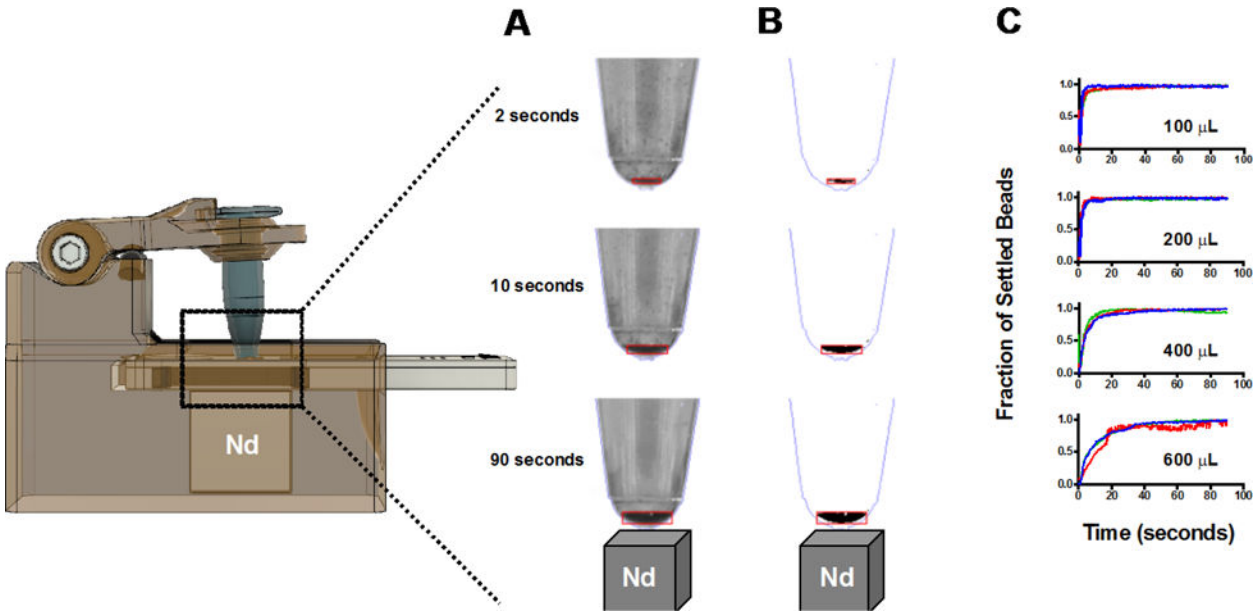

Figure 4.

Magnetic bead settling time experiments in 100 µL, 200 µL, 400 µL, and 600 µL whole lysed blood samples using time-lapse photography. **A)** The original image, **B)** image converted to a binary to identify only pixels containing clustered magnetic beads, and **C)** bead settled area integrated as a function of time.

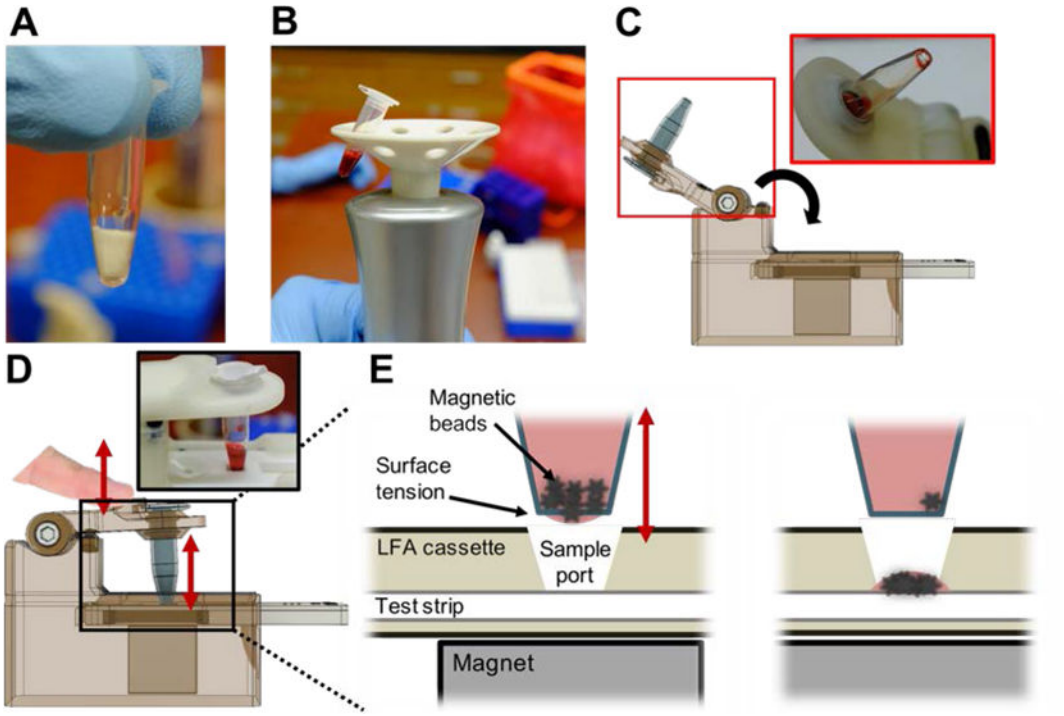

Figure 5.

The three-minute mBEADS workflow. A) The optimal single-use patient sample tubes are first loaded with hydrating lysis buffer and a whole blood sample and B) positioned onto a portable battery powered mixer for a 1 min mixing cycle. C) The mixed components are then moved by a transfer pipette to a sample deposition tube on board the mBEADS device in the ‘open’ position, the tube holder is then rotated into the ‘closed’ position over the LFA sample deposition port for a one-minute bead collection cycle. D) Beads are collected and retained at meniscus of the sample tube by surface tension are delivered to the LFA using the cantilever deposition action, where modified running buffer is added to the LFA test strip to elute HRP2 from the beads for downstream detection on the LFA.

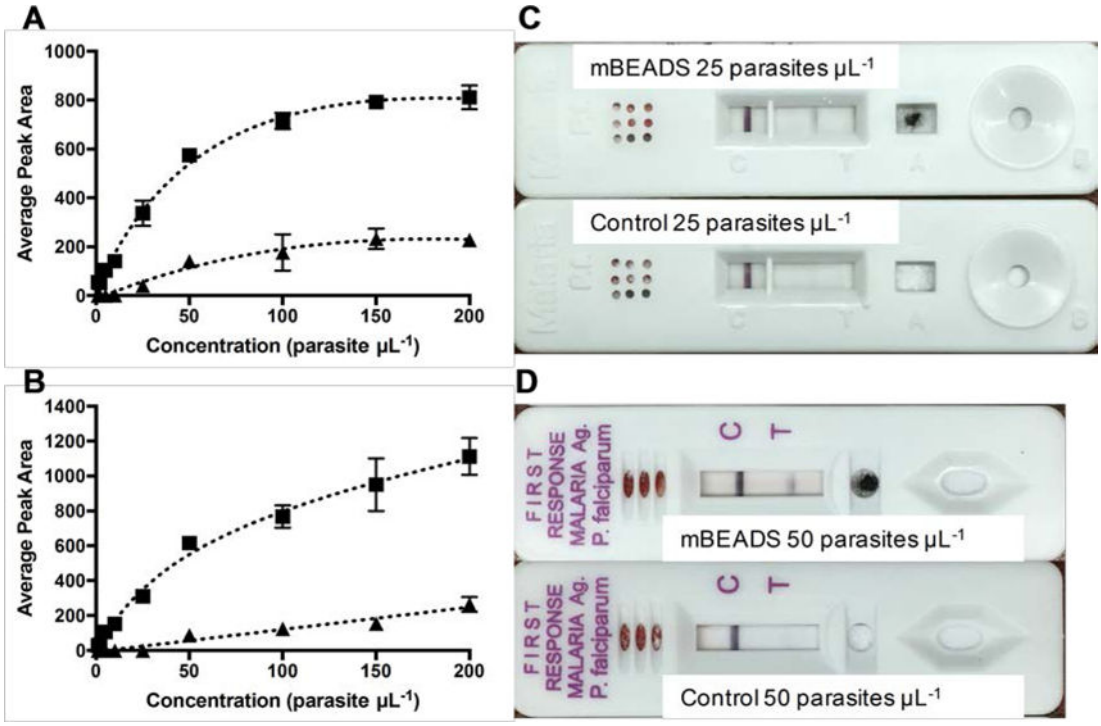

Figure 6.

mBEADS process enhances malaria LFA tests. A) Paracheck and B) First Response test line signals (T) after employing mBEADS to process 50 µL whole blood samples spiked to parasitemias ranging from 1 – 200 parasites μL^{-1} (squares). Control samples (triangles) were processed in parallel per manufacturer suggested protocol. Both graphs were fit with nonlinear best-fit curves for ease of view. The ICT-Pf dose-curve can be found in the supplemental section (Figure S3) Representative mBEADS processed and control LFAs for (C) Paracheck and (D) First Response tests are also provided.

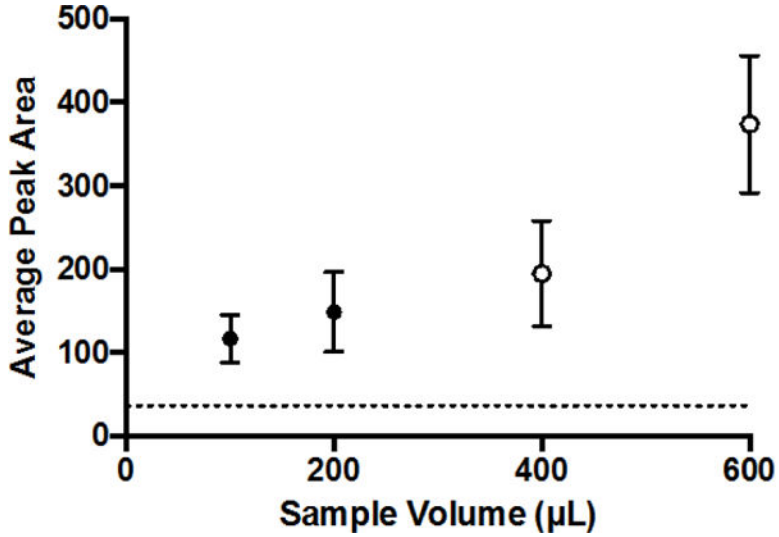

Figure 7.

Processing larger blood samples volumes further increases LFA signal. Utilizing the mBEADS methodology, LFA signal output was examined as a function of increasing blood sample volume (plotted as total sample volume following the 50:50 whole blood to lysis). Increased volumes of 1 parasite μL^{-1} whole blood samples were processed where 100 and 200 µL sample volumes were mixed for 1 min and bead settling time was set to 1 min (closed circles) and 400 µL and 600 µL total sample volumes (open circles) were conservatively mixed for 2 min and bead settling time was set to 2 min. A F-test concluded that no significant difference exists between the variances observed on the graph.

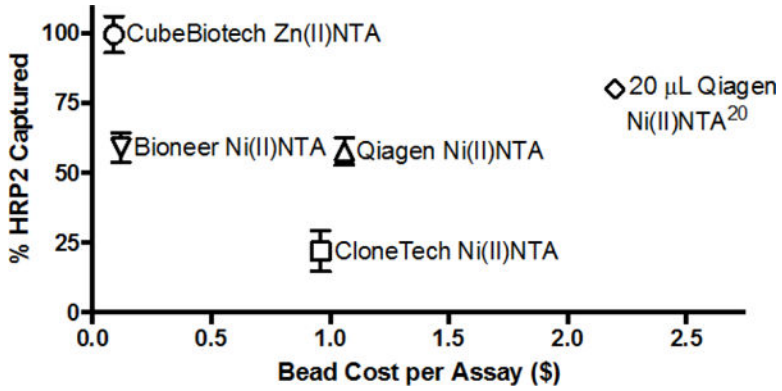

Figure 8.

HRP2 capture efficiency plotted against the cost of 5.5 mm^3 magnetically packed volumes of IMAC beads manufactured from four different manufacturers. A 1 min mixing cycle was used for Cube Biotech Zn(II)NTA and 10 min mixing cycles were used for the other three IMAC beads.

Table 1

Calculated LODs for Paracheck, First Response, and ICT-Pf with and without mBEADS.

LFA Type	LOD of Unenhanced LFAs		LOD of mBEADS Enhanced LFAs	
	[HRP2] (pM)	Parasites μL^{-1}	[HRP2] (pM)	Parasites μL^{-1}
Paracheck	36.0 ± 3.7	21.2 ± 2.2	4.1 ± 0.2	2.4 ± 0.1
First Response	52.0 ± 4.6	30.6 ± 2.7	2.2 ± 0.2	1.3 ± 0.1
ICT Pf	86.7 ± 22.1	51.0 ± 13.0	10.5 ± 0.9	6.2 ± 0.5