Supporting Information:

Metal Affinity-Enabled Capture and Release Antibody Reagents Generate a Multiplex Biomarker Enrichment System that Improves Detection Limits of Rapid Diagnostic Tests

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The contents of this document include supporting materials regarding the synthesis, purification, and characterization of the His_6-peptides used in this study. Also provided are studies that determined the optimal antibody-peptide conjugation conditions, the method used to calculate theoretical antibody bead coverage, the compatibility of CaR mAb reagents with the selected RDT, and a representative RDT image with and without the use of the biomarker enhancement strategy.

Supporting Materials and Methods: All Fluorenylmethyloxycarbonyl (FMOC)-protected amino acids, resins, peptide activators, and biotin were purchased from Advanced Chemtech and Aapptec. FMOC-Amido-dPolyethlyene glycol$_6$-acid (PEG$_6$) was purchased from Quanta Biodesign (OH, USA). Commercially available monoclonal antibodies were procured from Fitzgerald and Vista Diagnostics. All Octet RED96 biosensors were purchased from FortéBio (CA, USA). Sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC, No-Weigh™ Format) heterobifunctional crosslinker, 0.5 mL Zeba™ 7k MWCO spin desalting columns, MagnaRack™ magnetic separation rack, metal affinity Dynabeads® His-Tag Isolation and Pulldown magnetic beads, EZ-Link™ plus activated peroxidase kit, and Pierce™ Biotin Quantitation kit were purchased from ThermoFisher Scientific (MA, USA). Recombinant *plasmodium falciparum* and *plasmodium vivax pLDH* (rc*Pf*LDH and rc*Pv*LDH) were procured for CTK Biotech (CA, USA). ICT dual malaria RDTs were purchased from ICT diagnostics (Cape Town, South Africa). Whole pooled blood was obtained from bioreclamationIVT (TN, USA). *p*LDH ELISA kits were acquired from apDia (Belgium) and TMBone solution was purchased by Promega (WI, USA). Malaria strain *P. falciparum* D6, cultured in Vanderbilt University's BSL2 facilities and characterized by a trained microscopist (299,000 p/μL), was utilized to prepare mock clinical blood samples. Deionized water was used with a resistivity of 18 MΩcm. All other reagents, buffers, and solvents were used without further modifications from either Sigma-Aldrich or Fisher Scientific.

Instrumentation: Absorbance measurements were collected on a BioTek Synergy H4 plate reader (VT, USA). The His$_6$ Peptide was synthesized on a Peptide Machines Discovery-4 synthesizer (CA, USA). Peptides were purified with reverse phase high-powered liquid chromatography (HPLC) using a Waters Prep LC 4000 Preparative Chromatography System (MA, USA) containing a Waters 2487 dual wavelength detector. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry analysis was performed with an Applied Biosystems Voyager STR-4211 (MA, USA) instrument using a reflectron time-of-flight (TOF) mass analyzer. Real-time bio-layer interferometry (BLI) binding assays were performed with a ForteBio Octet RED96 bio-layer interferometer (CA, USA). Bead size and concentration was determined

using Countess® II automated cell counter procured from ThermoFisher Scientific (MA, USA). RDTs were read on a ESEQuant lateral flow reader procured from Qiagen (Germany).

Solid-Phase Peptide Synthesis of C-Peg$_6$-H-H-H-H-H-H-Peg$_6$-Biotin (His$_6$-Biotin): A cysteine rink amide resin was first loaded into a fritted peptide synthesis vessel. The resin was then swelled in dimethylformamide (DMF) as preparation for FMOC group removal through a 20 min treatment with 20% piperidine (v/v) in DMF. Afterwards, 3x molar excess (compared to the resin substitution) of FMOC-protected PEG$_6$ was coupled onto the resin overnight using 3x molar excess of hydroxybenzotriazole (HOBT) and 1,3-diisopropylcarbodiimide (DIC) activators. Following the overnight reaction, the resin was washed with DMF and methanol. For coupling procedures, 6x molar excess FMOC-amino acids were dissolved in a 6-dram vial containing 4 mL DMF, 6X molar excess HOBT, and 6x molar excess DIC. Coupling and deprotection reactions were performed on a plate shaker to agitate the resin, 60 min for coupling and 20 min for deprotection. Following the six sequential histidine additions, FMOC-protected PEG$_6$ was coupled onto the histidine-peptide overnight using the same conditions shown above. Finally, the peptide chain with PEG$_6$ spacer was biotinylated for one hour using 6x molar excess biotin, 6x molar excess HOBT, and 6x molar excess DIC.

Solid-Phase Peptide Synthesis of C-PEG$_6$-H-H-H-H-H-H-Ac (His$_6$): Outside the peptide synthesizer, a cysteine rink amide resin was first swelled in DMF and subsequently deprotected with 20% piperidine in DMF for 20 min. Afterwards, 3x excess of the FMOC-protected PEG$_6$ was coupled onto the resin overnight using 3x molar excess HOBT, 3x molar excess (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU), and 6x molar excess diisopropylethylamine (DIEA). After washing the resin with DMF and methanol, the resin was added to the peptide synthesizer to attach the histidine chain. For coupling procedures, FMOC-amino acids (0.4 M) dissolved in HBTU/DMF (0.2 M) were reacted with HBTU/DMF (0.4 M) and 40% v/v DIEA/DMF. Coupling reactions were performed for 60 min, while deprotection reactions were performed for 20 min. Finally, the peptide was acetylated for one hour using a 1:1:2 ratio of acetic anhydride:DIEA:DMF.

Peptide cleavage, purification, and characterization: After washing, the resin was incubated with 5 mL of 90:5:3:2 volume ratio of trifluoracetic acid:thioanisole:1,2-ethanedithiol:anisole for two hours while gently shaking to cleave the peptides which were then precipitated into cold diethyl ether and centrifuged (4°C, 1500 g, 5 min) four times to remove the ether and soluble organics. Next, the crude peptides were dissolved in 10 mL 1:1 water:acetonitrile and lyophilized. Afterwards, the peptides were further purified using reverse phase chromatography. The peptides were dissolved in 3 mL 1:1 water:acetonitrile and injected into a HPLC with a reverse-phase C18 column. Each dual wavelength-visualized HPLC product was collected in a separate purified fraction, lyophilized, and analyzed using MALDI-TOF (**Figure S1**). Micrograms of each peptide were dissolved in 1:1 water:acetonitritile, and 0.5 μL samples of the resulting solutions were spotted onto a MALDI plate. Immediately afterwards, 0.5 μL α-Cyano-4-hydroxycinnamic acid (CHCA, 20 mg/mL) matrix solution was mixed with the deposited peptide. Following the MALDI examination of each fraction, all pure fractions were combined, and stock solutions of 6 mM His_6-Biotin and 3 mM His_6 peptides were prepared and stored at -40°C.

Characterization of the CaR mAb reagents: A model anti-pLDH antibody (clone 19g7) was first functionalized with sulfo-SMCC at molar equivalents of 5x, 10x, 20x, 40x 60x, and 80x using the above conjugation protocol without alteration. A commercially available Pierce™ biotin quantitation kit was employed to determine the number of peptides per mAb. No modifications were made to the manufacturer's suggested microplate procedure for the quantitation of moles of biotin per mole of IgG protein. This assay was also performed for each mAb bioconjugate process control. The calculation of moles of biotin per mole IgG, led to the implementation of two correction factors: one based on the positive control and one based on its respective process controlled mAb. An Octet RED96, equipped with seven parallel ready-to-use Ni-NTA biosensors, was used to survey the impact that degrees of conjugation had on CaR mAb-pLDH binding. First, the Ni-NTA biosensor was introduced to a solution of 0.025% Tween 20 in phosphate buffered saline (PBST) for a 300 s equilibration step. Immediately after, the biosensors were moved to new wells for a loading step. Each well contained 3 μg/mL of mAbs functionalized with the previously indicated

series of sulfo-SMCC molar equivalents. Once loaded, a baseline was established by transferring the biosensors to wells containing PBST for another 60 s before being moved to new wells for an association step using 25 nM rcPfLDH. The binding phase shift maximum was collected at the end of 400 s. In addition, the same sulfo-SMCC conjugation conditions for each CaR mAb reagent was used to functionalize the respective antibodies with His$_6$ peptides, rather than a His$_6$-Biotin, to ensure that the terminal biotin did not interact with biosensor during the kinetic experiments. No significant difference was observed in the kinetic and equilibrium binding data between the two analogs (**Table S1**).

Methodology for calculating theoretical loading density: The theoretical CaR mAb bead coverage was calculated using an estimated CaR mAb docking area of 33.4 nm^2.[1] Docking area is the amount of space an immobilized mAb loaded on a bead will occupy. Given that each IgG possesses an approximate molecular weight of 150 kDa, this value can be used in conjunction with the mAb concentration and docking area per mAb to generate a total estimated docking area. The diameter of the magnetic His-Tag Isolation and Pulldown Dynabeads microparticles was measured to be 1.1 μm using a Countess II Automated cell counter (**Table S2**). Combining this information with the 1.325×10^7 beads/μL stock concentration resulted in a total bead surface area. Dividing the total CaR mAb docking area by the total bead surface area then multiplied by 100 yielded the theoretical bead coverage percentage.

Conjugation optimization of selected CaR mAb agents: All capture and release reagents that were found to bind both rcPfLDH and rcPvLDH, as well as anti-pLDH mAb clone 14c2, were selected to undergo complete conjugation optimization based on the results identifying the most favorable range of conjugation levels. 30 μL at 1 mg/mL of each of the six selected capture and release agents were conjugated using 10x, 20x, 30x, and 40x molar excess sulfo-SMCC along with 20x molar excess His$_6$-Biotin peptide. The antibodies conjugated at each condition were then evaluated for the ability to bind rcPfLDH. Kinetic experiments employing Ni-NTA biosensors (see previous experimental outline above) were performed to generate equilibrium constants, which were used as metric to determine optimal conjugation for each of the different capture and release agents.

In addition, the optimal sulfo-SMCC conjugation conditions for each CaR mAb reagent was used to functionalize the respective antibodies with a His_6 peptide. Kinetic binding experiments were then performed on the biotin-less CaR mAb reagents.

CaR mAb reagent RDT compatibility screening: Immunocomplexes were formed by incubating each identified pan anti-pLDH CaR reagent with native pLDH from culture. Stock parasite culture was diluted to 2,963 p/µL, which correlated to ~4,000 pM native pLDH protein. The culture dilution was aliquotted at 50 µL, and CaR agents were spiked in at 400 nM to achieve a 100x molar excess, or 100:1 ratio, when compared to pLDH. The samples were then gently mixed for 30 min on a plate shaker before spotting 5 µL of each onto a separate ICT Dual RDT and running according to the manufacturer's suggested protocol. In addition to complexed samples, pLDH controls without CaR mAbs were also prepared. Twenty minutes after sample addition the lateral flow assay strip was quantified using an ESEQuant Lateral Flow Reader.
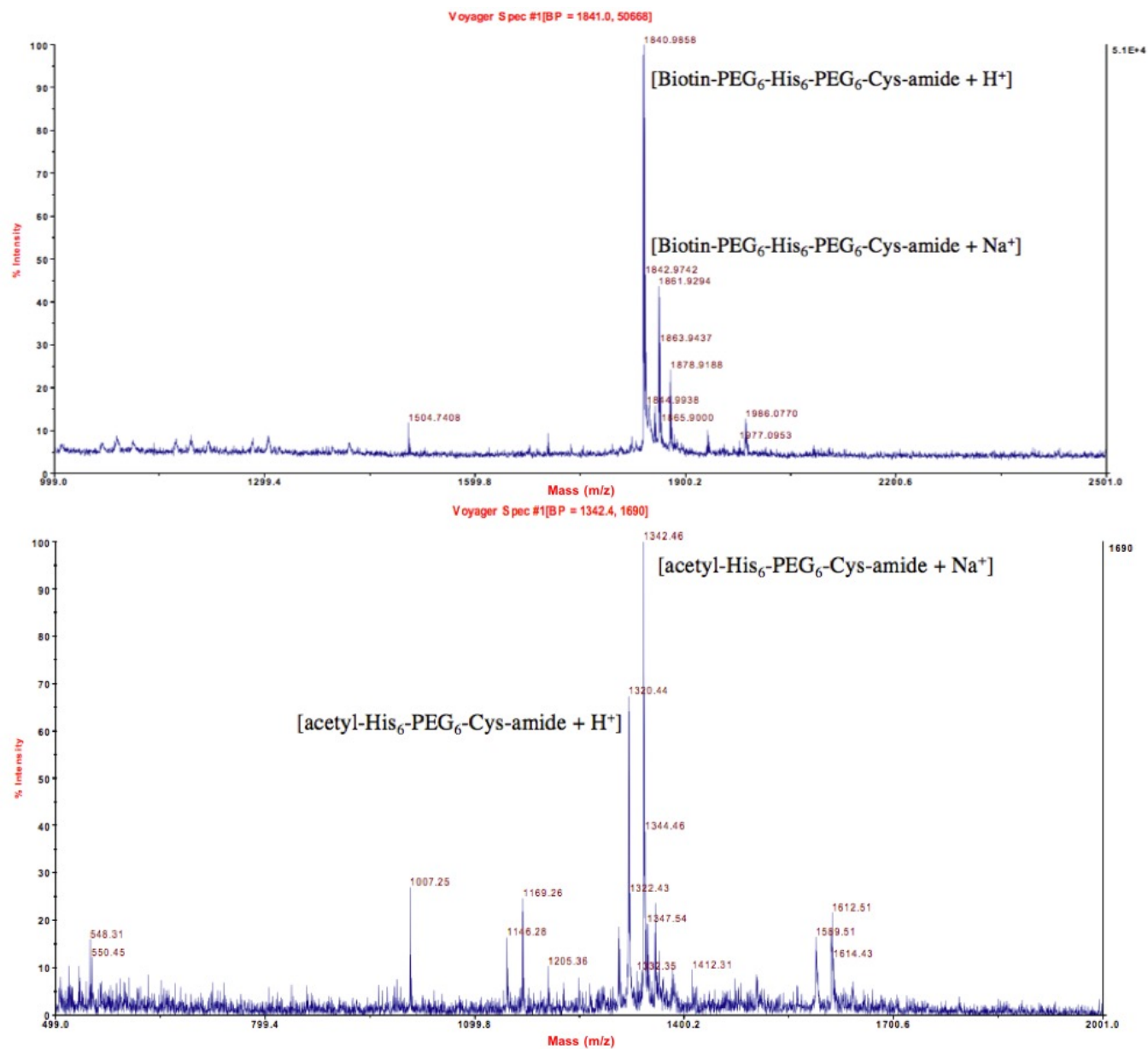
Figure S1. MALDI-MS spectrum of His_6-Biotin peptide (top) and His_6 peptide (bottom).
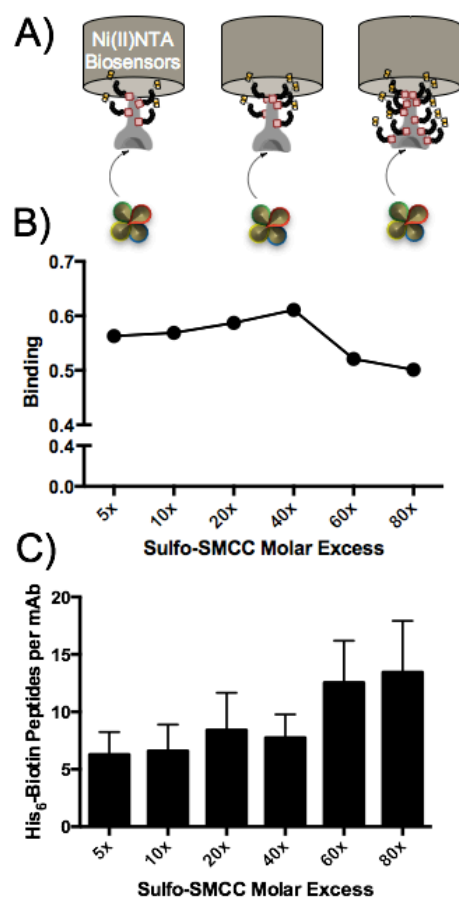
FIGURE S2: Impact of coupling strategy on antigen recognition. A model antibody, clone 19g7, was used to determine optimal conjugation conditions for the mouse IgGs employed in this study. A) An illustration of the experiment on Ni-NTA biosensors used to determine the B) impact of degree of mAb conjugation on CaR mAb reagent binding to rcPfLDH. C) Number of His6-Biotin peptides per mAb following the conjugation protocol as a function of molar equivalents of sulfo-SMCC.
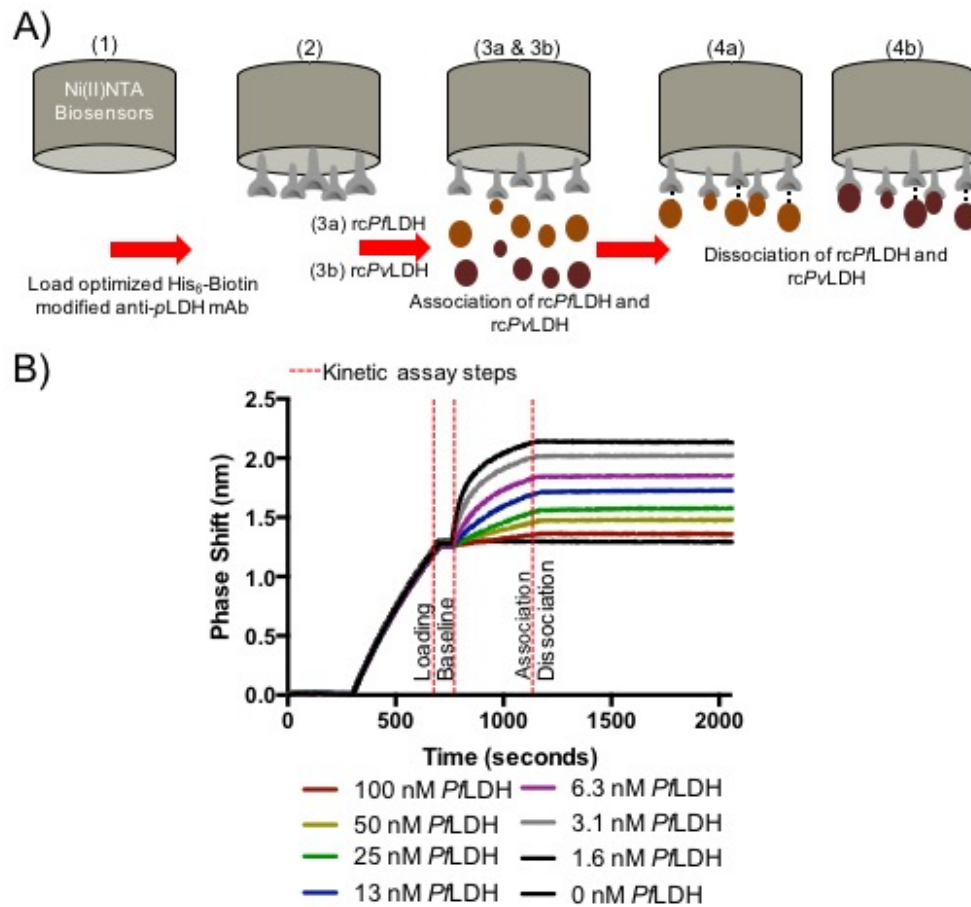
Figure S3. Bio-layer interferometry to evaluate binding performance of CaR mAb reagents. A) The process for kinetic experiments used to evaluate the binding affinity of CaR mAb reagents to rc*Pf*LDH and rc*Pv*LDH. B) Raw data from a BLI assay where CaR mAb 19g7 was loaded to biosensors for 400 s, baselined for 60 s in buffer, introduced to wells containing different *Pf*LDH concentrations ranging from 1.6 nM to 100 nM, and placed into buffer for a 900 s dissociation step. This data is globally fit to a 1:1 binding model to generate quantitative binding data (K_D, K_{on}, and K_{off}) between CaR mAb reagents and antigens.
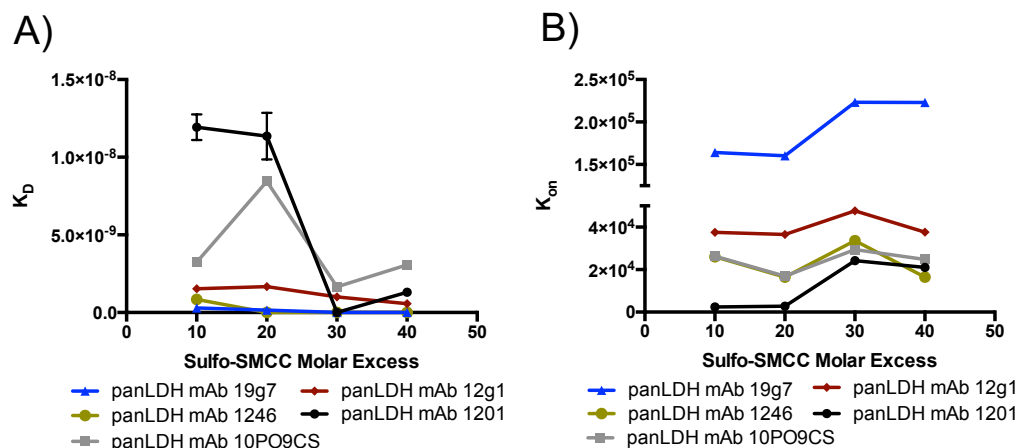
A)

B)

Figure S4. Pan *p*LDH CaR mAb reagent conjugation optimization. Selected Pan *p*LDH CaR mAbs that underwent a conjugation optimization study where they were modified with different molar equivalents of the crosslinking reagent, sulfo-SMCC. A BLI kinetic experiment was performed on each CaR mAb reagent conjugated with 10x, 20x, 30x and 40x sulfo-SMCC molar equivalents to evaluate their *p*LDH binding equilibrium (left), K_D, and association rate (right), K_{on}.
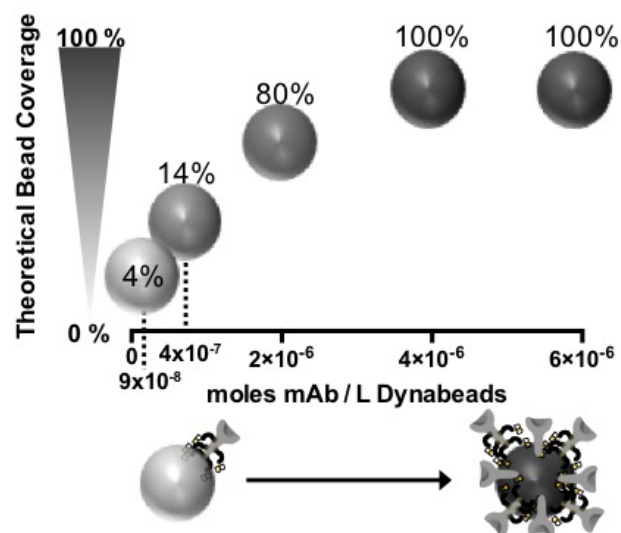
Figure S5: Antibodies per magnetic bead correlated to theoretical bead loading density. An illustration to visualize the correlation between moles CaR mAb/L Dyna-beads and theoretical bead coverage.
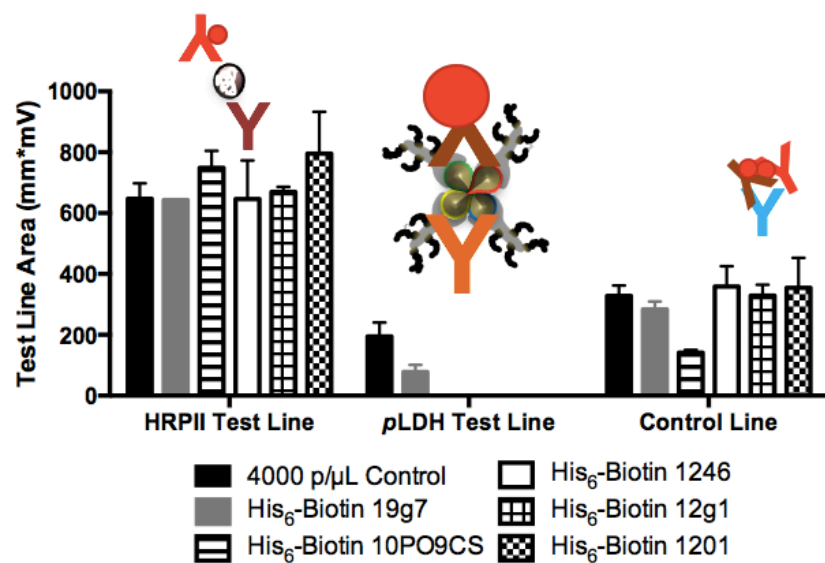
Figure S6: RDT compatibility study. Reflectance signal on the RDT HRPII, *p*LDH, and control test bands employing the capture and release strategy.
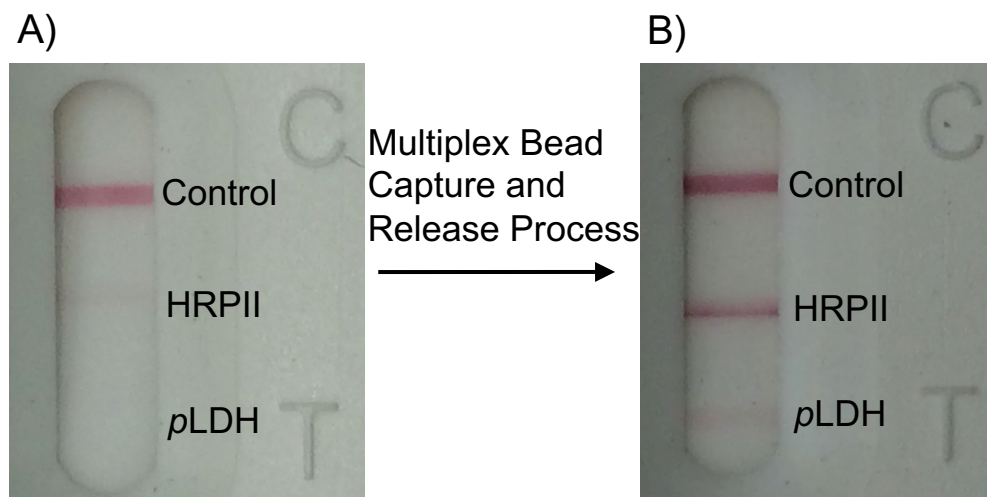
Figure S7: Visual enhancement of RDT employing multiplex biomarker enrichment strategy on ICT Dual malaria RDTs. A) A photograph taken 30 min after an RDT was run with 5 μL of 25 p/μL per manufacturer suggested protocol. B) A photograph taken 30 min after an RDT was run after processing 200 μL of 25 p/μL per the multiplex bead capture and release protocol.

Table S1. Determining if the terminal biotin on the His$_6$-Biotin peptide interacts with the streptavidin-based NiNTA biosensor during kinetic experiments. Quantitative binding data between CaR mAbs modified with a His$_6$ peptide, instead of His$_6$-Biotin and rc*Pf*LDH and rc*Pv*LDH. The different antibodies are distinguished by their commercial IgG clonality label and should be compared to kinetic data reported in the manuscript.

His$_6$- modified mAb	rcPfLDH		rcPvLDH	
IgG clone	k_{on} (1/Ms)	K_D (M)	k_{on} (1/Ms)	K_D (M)
10PO9CS	4.49E+04	4.40E-10	7.57E+03	2.75E-9
15f10	4.13E+04	1.43E-08	NB	NB
14c2	NB	NB	NB	NB
19g7	2.30E+05	<1.0E-12	1.38E+05	1.64E-10
12g1	4.59E+04	2.26E-10	6.47E+04	1.44E-09
6c9	2.92E+04	7.58E-10	NB	NB
1201	1.28E+04	4.95E-09	6.04E+02	4.72E-08
1246	3.33E+04	<1.0E-12	1.46E+03	1.48E-08
10PO9F (*Pf*LDH mAb)	7.88E+03	3.13E-08	NB	NB

Table S2. Characterization of magnetic beads used in this study. Data generated from the Countess II cell counter used to determine the concentration and size of the magnetic IMAC beads used in this study. The stock bead solution had to be diluted 1 to 10,000 to generate the bead count employing this instrument.

Average Count (beads/mL)	Dilution On Countess II	Average bead count in stock bead solution (beads/mL)	Average Size (μm)
2.65E+06 ± 1.75E+5	1/10,000	2.65E+10 ± 1.75E+09	1.10 ± 0.06

References:

1.   Cytodiagnostics, Gold, Silver, and Gold NanoUrchin Conjugate Technical Information.