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Simultaneous capture and sequential detection of two malarial biomarkers on magnetic microparticles

Christine F. Markwalter [1], Keersten M. Ricks [1,2], Anna L. Bitting, Lwiindi Mudenda, David W. Wright *

Department of Chemistry, Vanderbilt University, Station B 351822, Nashville, TN 37235, USA

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ABSTRACT

We have developed a rapid magnetic microparticle-based detection strategy for malarial biomarkers *Plasmodium* lactate dehydrogenase (*p*LDH) and *Plasmodium falciparum* histidine-rich protein II (*Pf*HRPII). In this assay, magnetic particles functionalized with antibodies specific for *p*LDH and *Pf*HRPII as well as detection antibodies with distinct enzymes for each biomarker are added to parasitized lysed blood samples. Sandwich complexes for *p*LDH and *Pf*HRPII form on the surface of the magnetic beads, which are washed and sequentially re-suspended in detection enzyme substrate for each antigen. The developed simultaneous capture and sequential detection (SCSD) assay detects both biomarkers in samples as low as 2.0 parasites/μl, an order of magnitude below commercially available ELISA kits, has a total incubation time of 35 min, and was found to be reproducible between users over time. This assay provides a simple and efficient alternative to traditional 96-well plate ELISAs, which take 5–8 h to complete and are limited to one analyte. Further, the modularity of the magnetic bead-based SCSD ELISA format could serve as a platform for application to other diseases for which multi-biomarker detection is advantageous.

1. Introduction

Enzyme-linked immunosorbent assays (ELISAs) are the gold standard laboratory technique for quantitative and qualitative protein detection, serving as both powerful research tools and clinical diagnostics. These highly sensitive assays are typically performed in a microtiter plate, utilizing surface-bound antigen or antibody to bind a protein analyte and enzyme-conjugated, target-specific antibodies for detection. Although traditional singleplex ELISAs are laboratory "workhorses" for sensitive and specific protein detection, they require 5–8 h for completion and several incubation steps to ultimately develop signal. Further, conventional ELISAs are limited to detecting just one analyte from a single sample.

While traditional ELISAs are useful for diagnoses arising from one biomarker, diseases requiring multi-analyte detection to identify or inform treatment have led to the development of multiplexed immunoassays. A multiplexed immunoassay utilizes the same "sandwich" format (capture antibody, sample, detection antibody) as a conventional singleplex ELISA, except the former usually adopts fluorescent or chemiluminescent reporter systems rather than amplification of a colorimetric substrate by enzymes [1]. Two common formats for multiplexed immunoassays include planar arrays and bead-based suspension assays [2]. In typical, commercially available planar arrays (Quansys, MSD®), microliter volumes of capture antibodies for multiple protein biomarkers are printed discretely onto two-dimensional supports, such as slides or microtiter plates, using a high-resolution printer. The functionalized supports are then treated with sample, followed by reporter-labeled antibody. Signal is detected using a high-resolution scanner or fluorescence microscope [1]. In addition to multiplexing capabilities, planar micro-array immunoassays benefit from ambient analyte theory. According to ambient analyte theory, reducing the concentration of capture antibodies results in increased antibody binding site occupancy and thus higher assay sensitivity [3,4]. However, these benefits are often off-set by mass transport limitations [1].

In contrast to planar arrays, bead-based suspension immunoassays are advantageous because they overcome mass transport limitations via active mixing throughout the liquid sample [5]. In a typical bead-based suspension immunoassay (Luminex™, Bio-PlexPro™, Cytometric Bead Arrays), fluorescent microbeads are functionalized with capture antibodies, mixed with a sample, and subsequently mixed with fluorescently-tagged

* Corresponding author.
E-mail address: david.wright@vanderbilt.edu (D.W. Wright).
[1] Authors contributed equally to this work.
[2] Present address: US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA.

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detection antibodies, which allow for analyte detection via flow cytometric methods. Multiplexing capabilities arise when target-specific antibodies are functionalized to microbeads with varying fluorescent signatures distinguishable by flow cytometry [6].

There are several disadvantages to current multiplexed immunoassays. Both planar and bead-based immunoassays require laboratory infrastructure beyond that needed to perform singleplex conventional ELISAs; planar micro-array assays require high-resolution fluorescence scanners, and bead-based immunoassays require flow cytometric instrumentation for detection [1]. Further, planar micro-arrays require several addition, wash, and incubation steps totaling up to 3 h [7]. Commercially available bead-based suspension assays often require 3–4 h for completion, up to 1 h dedicated to the detection step [8]. To address these pitfalls, we have developed a magnetic bead-based ELISA in which two biomarkers are simultaneously captured and sequentially detected in less than 1 h with no laboratory infrastructure beyond what is required to perform a conventional singleplex well-plate ELISA.

We applied the developed magnetic bead-based ELISA to the detection of two malarial biomarkers: (1) *Plasmodium* lactate dehydrogenase (*p*LDH), and (2) *Plasmodium falciparum* histidine-rich protein II (*Pf*HRPII). Malaria, a mosquito-borne infectious disease caused by *Plasmodium* protozoan parasites, claimed over 400,000 lives in 2015 [9]. Accurate diagnosis of malaria is imperative for defining disease prevalence and distribution as well as monitoring impact of interventions. Furthermore, identification and proper treatment of asymptomatic cases (<200 parasites/μl), which serve as transmission reservoirs, are critical for eliminating the disease [10]. An assay that detects both *p*LDH and *Pf*HRPII is beneficial for several reasons. First, *p*LDH is a parasite metabolic enzyme, so it is present for infections resulting from any of the five species of malaria known to infect humans, whereas *Pf*HRPII is only present in *P. falciparum* infections [11,12]. Thus, an assay that detects both biomarkers can differentiate between *P. falciparum* and non-*falciparum* infections, a distinction that determines proper treatment [13]. Second, *Pf*HRPII remains in host circulation for up to one month, whereas *p*LDH is known to clear within 24 h post parasite clearance, so a dual assay can distinguish resolved and active *P. falciparum* infections [14]. The magnetic bead-based simultaneous capture and sequential detection (SCSD) ELISA for *p*LDH and *Pf*HRPII would not only inform patient management, but also allow for more efficient and sensitive *P. falciparum* and non-*falciparum* epidemiology and transmission studies. The presented assay design is modular and can be applied to any set of two biomarkers provided validated antibody pairs are available.

2. Experimental

2.1. Reagents and materials

Dynabeads® MyOneTM Streptavidin T1 beads were purchased from Life Technologies (Cat #65601). Recombinant *P. falciparum* lactate dehydrogenase (rc*Pf*LDH) and recombinant *Plasmodium vivax* lactate dehydrogenase (rc*Pv*LDH) were purchased from CTK Biotech (Cat #A3005, #A3004). *P. falciparum* D6 strain was cultured in the lab. *P. falciparum* W2, Benin 1, and PH1 reference strains were obtained from the Foundation for Innovative New Diagnostics (FIND). Anti-*Pf*HRPII capture and detection antibodies were purchased from Abcam (ab9203 and ab30384). Pan-specific α-*p*LDH antibodies were purchased from AccessBio, Fitzgerald, and Vista Diagnostics (Table S1). BluePhos® Microwell Phosphatase substrate was purchased from KPL (#50-88-02), and TMB One was purchased from Promega (G7431). The ELISA kit for *p*LDH was purchased from SD Bioline, S. Korea (05EK40), and the ELISA kit for *Pf*HRPII was purchased from Cellabs, Australia (KM2).

2.2. pLDH antibody pair screen

Capture and detection antibodies were screened for use in the *p*LDH on-bead ELISA. Briefly, 64 antibody pairs were tested (8×8 matrix) in a checkerboard 96-well plate ELISA format. Each of the 8 antibodies was conjugated to alkaline phosphatase (AP) for detection (Abcam, ab102850). 100-μl solutions of 1 μg/ml unmodified anti-*p*LDH IgG were incubated for one hour in Immulon 2 HB 96-well plates (Thermo Scientific #3455). The plates were then washed 3 times with 1 × phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST). Next, 250 μl of 5% w/v bovine serum albumin (Fisher BP1600) in PBST was incubated for 2 h in each well. The plates were then washed 3 times with PBST. Samples consisting of 0 and 100 parasites/μl *P. falciparum* D6 culture or 0 and 500 pM rc*Pv*LDH were added to the plates in triplicate in PBST containing 0.1% BSA and incubated for 2 h. The plate was then washed 5 times with 1 × tris buffered saline (TBS) containing 0.1% Tween-20 (TBST). Next, 100 μl of 0.5 μg/ml of detection antibodies in TBST with 0.5% BSA was added to each well, and the plates were incubated for 1 h while protected from light. The plates were then washed 5 times with TBST, and 100 μl of BluePhos® Microwell Phosphatase Substrate was added to each well and incubated for 20 min while protected from light. The absorbance was measured at 620 nm using a Synergy H4 microplate reader. Signal-to-noise ratios were determined for each pair and antigen.

2.3. Blood sample preparation

Pooled human whole blood (Bioreclamation IVT, HMWBCPD) was spiked with D6 *P. falciparum* culture (at 18,450 parasites/μl) to the desired parasitemia. An equal volume of 2 × lysis buffer (100 mM potassium phosphate pH 8.0, 600 mM NaCl, 250 mM imidazole, 2% Triton-X-100) was then added, and the lysed blood was filtered through glass wool in a plastic syringe.

2.4. Preparation of mAb-functionalized magnetic beads

Target-specific antibody-functionalized beads were prepared as reported previously [15]. Briefly, α-*p*LDH (Vista, 19g7) or α-*Pf*HRPII (Abcam, ab9203) antibodies were biotinylated with EZ-Link NHS-PEG4-Biotin, No-Weigh Format (Thermo Pierce #21329) in PBS with a 20× excess of NHS-PEG4 Biotin. Remaining NHS-PEG4 biotin was removed using Zebra Spin Desalting Columns with a 7 K molecular weight cut-off (Thermo Pierce #89882). Next, 5 mg of Dynabeads® MyOne™ Streptavidin T1 was washed 3 times with PBS before incubating for 30 min with 500 μl of 0.4 μg/ml of biotinylated antibody in PBS. The beads were then washed 3 times with PBS and blocked with excess D-biotin in PBS for 30 min. Finally, the beads were washed 3 times and re-suspended in 500 μl of PBS with 0.01% Tween-20.

2.5. On-bead ELISA for pLDH

Solutions (200 μl) of parasitized lysed whole blood were placed in a Fisherbrand Flat-bottom PS 96-well plate (#12565501). Four μl HAMA blocker (Fitzgerald 85 R-1001), 10 μl of α-*p*LDH (19g7) magnetic beads, and 1.57 μl of 1201:AP (1.27 mg/ml) were added to each well and incubated on an orbital shaker for 15 min. Using a MagWell™ Magnetic Separator (EdgeBio #57624), the beads were separated from the supernatant and washed with 200 μl PBST. As a second wash, 100 μl PBST was added to the beads, which were then moved to new wells. Next, 100 μl BluePhos® Microwell Phosphatase Substrate was added to each well containing beads, and the plate was incubated for 15 min while protected from light. The supernatant was removed, and signal was measured by absorbance (620 nm) on a plate reader.