POINT-OF-CARE IMMUNOASSAY CARDS FOR SAMPLE-TO-RESULT DIFFERENTIAL DIAGNOSIS OF ACUTE FEVER

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ABSTRACT

We present air-driven microfluidic cards that perform sample processing and parallel immunoassays for antigens and IgM antibodies. The disposable cards are based on a flow-through membrane immunoassay carried out on porous nitrocellulose, which provides rapid diffusion for short assay times and a high surface area for visual detection. Malarial antigen, *Plasmodium falciparum* HRPII antigen, showed a limit of detection of 20-30 ng/mL spiked into fetal bovine serum. Detection of IgM antibodies to *Salmonella* Typhi, from clinical plasma samples, correlated closely with bench-top tests. All reagents were stored dried on card; only sample and buffer were required to run the test.

KEYWORDS: air-driven microfluidics, immunoassay, nitrocellulose, multiplexing

INTRODUCTION

In many low-resource settings (LRSs) multiple diseases that cause similar clinical symptoms are endemic. There is a general need for diagnostic tests appropriate for LRSs[1-3], and an additional need for multi-analyte diagnostics that can differentiate between diseases that present with similar clinical symptoms. The work here was part of a larger effort to develop a microfluidic point-of-care system, the DxBox, for sample-to-result differential diagnosis of infections that present with high rapid-onset fever. Here we describe a platform that performs parallel immunoassays for antigens and IgM antibodies as markers of current infection. Assay reagents were stored on card in dry form requiring only sample and buffer to run each test.

EXPERIMENTAL

The DxBox cards performed a sandwich immunoassay on a nitrocellulose membrane by flowing reagents and sample transversely through the membrane using air pressure. The flow-through membrane immunoassay (FMIA) had the benefits of short assay times and high capture surface area for non-amplified visual detection, similar to lateral flow tests. The FMIA also utilized multiple wash steps to rinse away non-specific signal, similar to the laboratory standard, enzyme-linked immunosorbent assays (ELISA).

The microfluidic cards (Figure 1) were fabricated from multiple layers of laser-cut laminates with the membranes and dry reagent pads integrated into the cards. The card required many laminate layers (23 including a valve layer) and pick-and-place steps to integrate the air vents, the assay membrane, blood filtration membrane, and dry reagent pads. Possible methods to decrease the number of layers include injection molded parts with features on both faces, or confining membranes to a single plane within the card. The assay membrane was nitrocellulose with a nominal pore diameter of 450 nm, which provided rapid diffusion of analyte/label molecules to the membrane surface. Capture molecules were deposited on the assay membrane and dried before the membrane was integrated into the cards. Antibody labels conjugated to gold nanoparticles (Au-Ab) were dried on polyester conjugate pads in the presence of trehalose.

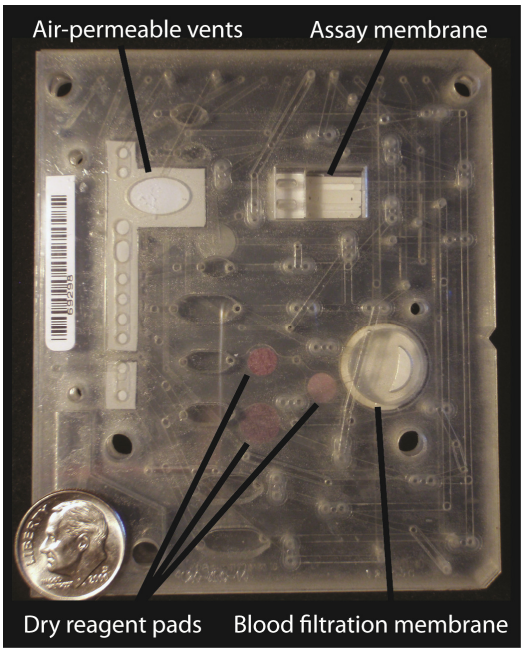

Figure 1: Photograph of the multi-layer microfluidic card.

Figure 2 describes the sample processing and assay steps performed in the DxBox cards. A blood sample was added to the card and drawn through a plasma extraction membrane to remove blood cells. The plasma was aliquoted into two sample volumes. The IgM sample required further processing; it was diluted with buffer and mixed with protein-G beads to remove IgG antibodies (assay interferent). Then, the antigen sample and processed IgM sample were pushed transversely through the assay membrane where immobilized reagents captured the target analytes (malaria antigen pfHRPII and IgM to *S.* Typhi). Each target was labeled with reconstituted Au-Ab.

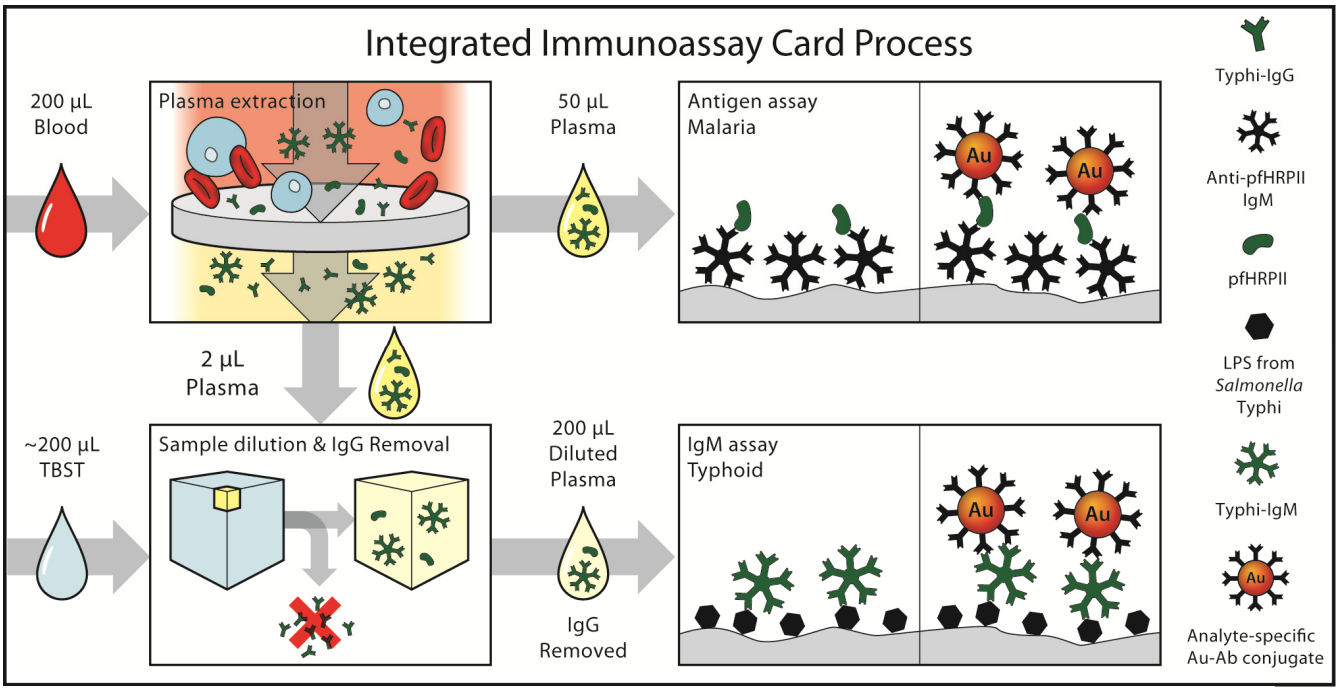

Figure 2: Process flow for the sample processing and FMIA performed by each microfluidic card.

The DxBox cards were actuated by a system of air pumps with possible output pressures of 1 psi, atmosphere or -1 psi. In contrast, most microfluidic systems are driven by syringe pumps which provide direct control over fluid flowrate and delivered volume. To provide reproducible flowrates and aliquoted volumes, we designed several card features that use air-permeable vents to enable pneumatic card operation. 1) We developed an on-card pressure regulator analogous to an electrical voltage divider to allow on-demand selection of driving pressures from a single air pump. 2) Sample, wash, and reagent volumes were aliquoted by drawing fluids into vented chambers of defined volume, then each fluid was sequentially delivered by applying pressure to the vent. 3) The IgM assays required a batch mixer to carry out sample dilution and a bead-based IgG removal step. We developed a batch mixer that uses bubbles to drive mixing in a chamber with a vented ceiling.[4] 4) Pneumatic fluid control inherently led to air gaps between fluids, so a vent was added to remove air between each fluid delivery step.

To quantify the result of each test, the assay membrane was imaged and analyzed to calculate the assay signal for that test. Spot signals and background signals were quantified as the green channel intensity of RGB images. The raw intensities of the image were normalized to give a scale representing pure white as 0 and pure black as 1. The assay signal presented for each spot was defined as the normalized intensity of that spot minus the normalized intensity of the local background.

RESULTS AND DISCUSSION

Antigen detection in the DxBox cards was demonstrated by capturing pfHRPII antigen, indicative of a dangerous malaria infection. A range (0 – 100 ng/mL) of recombinant pfHRPII antigen was spiked into fetal bovine serum and tested on card (Figure 3). The membrane images show darker sample spots with increasing pfHRPII concentration. The limit of detection (LOD) found was 20-30 ng/mL. We previously presented an FMIA card driven by syringe pumps that utilized dry reagents, and demonstrated pfHRPII detection with an LOD of 10-20 ng/mL.[5] The LOD for laboratory ELISA is ~4 ng/mL, which corresponds to ~50 parasites/µL.[6]

IgM specific to *S.* Typhi was detected from a panel of clinical plasma samples (Figure 4). The images show example membranes from known

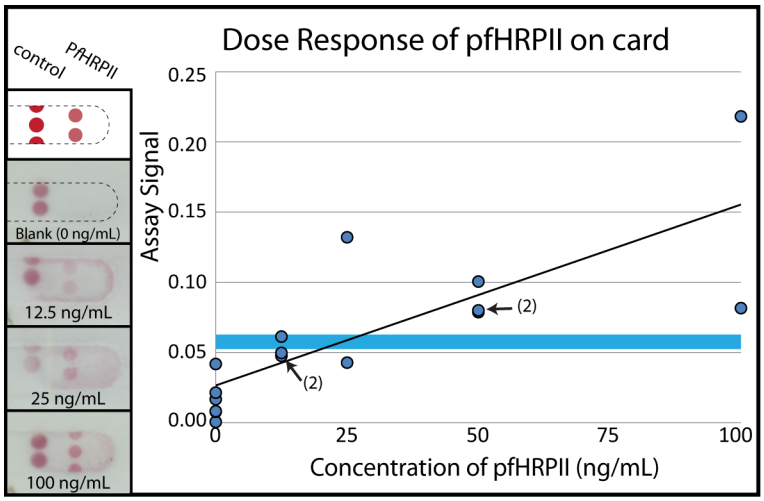

Figure 3: Recombinant pfHRPII antigen was spiked into fetal bovine serum at a range of concentrations. Measured limit of detection was 20-30 ng/mL (blue bar).

positive and negative samples. The membrane images also illustrate the full array of spots detected during the IgM assays: a process control (P.C.), which confirmed exposure to detection labels, an endogenous control (E.C.), which indicated a human plasma or blood sample was tested, and signal indicating *S.* Typhi IgM. The quantified signal for *S.* Typhi IgM detected using the FMIA on the DxBox cards (blue) correlated well with the bench-top FMIA (green) and ELISA (purple) results across the clinical panel.

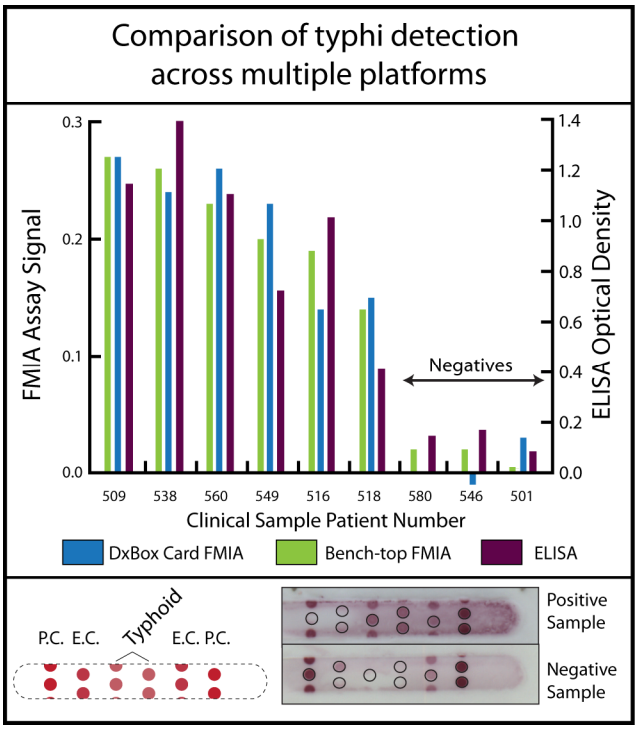

Figure 4: IgM indicative of S. Typhi demonstrated comparable signal response in the DxBox cards to bench-top FMIA and ELISA, across a panel of clinical samples. Images show the spot patterns for IgM capture including a process control (P.C.) and endogenous control (E.C.).

CONCLUSIONS

Controlling the flowrate of fluids actuated by air was a significant challenge due to variations in fluid viscosity, the presence of bubbles, and variations in channel and via fabrication. Flowrate variations affected the time each reagent spent in contact with the assay membrane, which for the target membrane contact times chosen impacted pfHRPII quantification but had little impact on *S.* Typhi IgM detection. Diffusion in the FMIA is very rapid, so the fastest sample-to-result time can be achieved by decreasing the target membrane contact time to be comparable with the time to bind sufficient analyte from the sample. Extending the target contact times make the assay less sensitive to variations in actual contact time and provided more reproducibility, however longer contact times extend the sample-to-result time.

The DxBox immunoassay cards integrate sophisticated sample preparation (including plasma filtration from whole blood, sample and reagent aliquoting for the two parallel assays, sample dilution, and IgG removal for the IgM assay) with multi-step assay protocols into a single, self-contained card. The cards demonstrate a true sample-to-result system which stored all reagents on card in dry form and required only sample and buffer be added to run the tests. For the detection of a malaria antigen and IgM antibodies to *S.* Typhi, the disposable immunoassay cards demonstrated performance comparable to laboratory assays.

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REFERENCES:

[1] S. R. Jangam, D. H. Yamada, S. M. McFall and D. M. Kelso, *J. Clin. Microbiol.*, 2009, 47, 2363-2368.
[2] A. W. Martinez, S. T. Phillips, G. M. Whitesides and E. Carrilho, *Analytical Chemistry*, 2010, 82, 3-10.
[3] P. Yager, G. J. Domingo and J. Gerdes, *Annual Review of Biomedical Engineering*, 2008, 10, 107-144.
[4] K. G. McKenzie, L. K. Lafleur, B. R. Lutz and P. Yager, *Lab Chip*, 2009, 9, 3543-3548.
[5] D. Y. Stevens, C. R. Petri, J. L. Osborn, P. Spicar-Mihalic, K. G. McKenzie and P. Yager, *Lab Chip*, 2008, 8, 2038-2045.
[6] C. M. Kifude, H. G. Rajasekariah, D. J. Sullivan, V. A. Stewart, E. Angov, S. K. Martin, C. L. Diggs and J. N. Waitumbi, *Clinical and Vaccine Immunology*, 2008, 15, 1012-1018.

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