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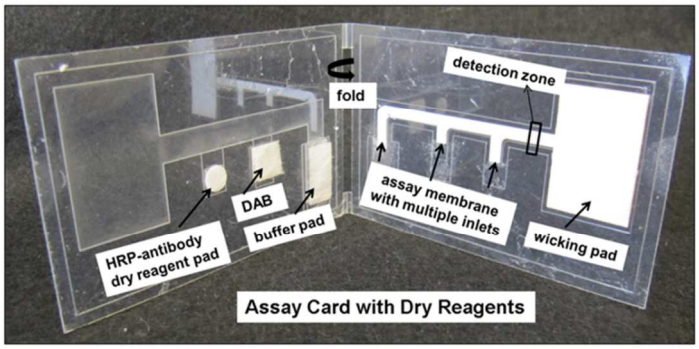
A folding 2DPN device with incorporated dry reagents for automated ELISA



Long-term dry storage of an enzyme-based reagent system for ELISA in point-of-care devices

Sujatha Ramachandran*, Elain Fu, Barry Lutz, and Paul Yager

Abstract:

Lateral flow devices are commonly used for many point-of-care (POC) applications in low-resource settings. However, they lack the sensitivity needed for many analytes relevant in the diagnosis of diseases. One approach to achieve higher sensitivity is signal amplification, which is commonly used in laboratory assays, but uses reagents that require refrigeration and inherently requires multiple assay steps not normally compatible with POC settings. Enzyme-based signal amplification, such as the one used in ELISA, could greatly improve the limit of detection if it were translated to a format compatible with POC requirements. A signal-amplified POC device not only requires the reagents to be stored in a stable form, but also requires automation of the multiple sequential steps of signal amplification protocols. Here, we describe a method for the long-term dry storage of ELISA reagents: horseradish peroxidase (HRP) conjugated antibody label and its colorimetric substrate diaminobenzidine (DAB). The HRP conjugate retained ~80% enzymatic activity after dry storage at 45 °C for over 5 months. The DAB substrate was also stable at 45 °C and exhibited no detectable loss of activity over 3 months. These reagents were incorporated into a two-dimensional paper network (2DPN) device that automated the steps of ELISA for the detection of a malarial biomarker. These results demonstrate the potential of enzyme-based signal amplification for enhanced sensitivity in POC devices for low resource settings.

Keywords: HRP-antibody dry preservation; dip stick; immunoassay; paper microfluidics; lateral flow test; chemical signal amplification

Introduction

Point-of-care (POC) devices that are accurate, robust, low cost, rapid, easy-to-use, equipment-free and disposable are in great demand for the diagnosis of diseases in low-resource settings [1, 2]. Existing lateral flow tests (LFTs) are simple wicking-based devices that, typically, use colloidal gold for detection of proteins or antibodies and meet many of the requirements for low-resource settings. However, they lack the high sensitivity needed for many analytes and can lead to misdiagnosis [3, 4]. A main source of this lack of sensitivity arises from the fact that the most common visually-observable labels (e.g., gold nanoparticle labels) are not particularly sensitive. Labels such as latex beads and carbon black have been substituted for gold in LFTs and been reported to have improved sensitivity [5, 6]. Fluorescent labels have been used for greater sensitivity[7], but at the expense of adding complexity and cost. Additionally, there have been reports of chemical signal amplification using silver and gold enhancement for overcoming sensitivity limitations [8, 9]. Enzyme-based signal amplification, for example, using the horseradish peroxidase (HRP) system commonly implemented in ELISA, could greatly improve limits of detection (LOD) if it could be made compatible for use in POC devices. The two main challenges to this are 1) the long-term stability of the HRP conjugated antibody and its substrate and 2) the capability of the POC device to perform the sequential multistep processes needed for the enzyme-based signal amplification without added user steps.

Methods for stabilizing proteins and enzymes in dry form using sugars and sugar alcohols are widely used in the pharmaceutical industry. The most common method of making solid proteins is lyophilization [10]. Trehalose, a non-reducing disaccharide can form protein-stabilizing glass and is the most common sugar used in the dry preservation of biomolecules [11-17]. Transition metal ions formulated in sugars have been reported to stabilize enzymes [18-20]. The stabilization of several enzymes including HRP, using a combination of sugar alcohols and cationic polymers or zinc ions was reported and have found application in extending the shelf-life of diagnostic alcohol kits [21, 22]. There have been reports on methods of stabilization of HRP immobilized onto various matrices like SiO_2 [23] and polyacrylonitrile [24] for use in analytical systems as biosensors. One patent describes a method of freeze-drying peroxidases including HRP in glass tubes in the presence of polyvalent ions, most preferably ferrous ion, and long-term storage at 37 °C (US patent 4331761). However there have been no further reports incorporating these methods into a diagnostic device. We adapted the method described in this patent that uses ferrous ions to stabilize the HRP for use in a diagnostic device. Several significant modifications were made in our system. First, we used trehalose instead of mannitol and sucrose in our reagents. Second, we chose vacuum-drying instead of lyophilization. And third, we used glass fiber pads for storing the reagents in

contrast to storing in glass tubes. Finally, our reagents were stored at an elevated temperature of 45 °C as compared to 37 °C in the patent.

There have been a few reports on the incorporation of HRP enzyme-based signal amplification in a lateral-flow-like device [25-27]. These methods combined the high sensitivity of the enzymatic amplification with the rapid lateral flow format; however they still required many timed user steps that make them less compatible with POC testing. In addition, these reports relied on fresh wet reagents and did not address the long-term stability of HRP or its substrates.

We recently reported on a two-dimensional paper network format (2DPNs) that uses shaped paper with multiple inlets to "program" automated multistep assays sequences while requiring only a single user activation step [28]. In this paper, we describe a method for the long-term elevated-temperature dry storage of reagents for enzyme-based signal amplification – antibodies conjugated to horseradish peroxidase (HRP) and the HRP substrate diaminobenzidine (DAB). Further, we incorporated the dry reagents into a 2DPN device and demonstrated an automated ELISA for detection of a malarial biomarker *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP2) in a sandwich immunoassay. *Pf*HRP2 protein is secreted by the parasite into the host bloodstream and can be detected in serum, plasma and cerebrospinal fluids [29, 30]. The dry preservation of enzyme-based signal amplification reagents could have value for a number of applications that use enzyme-based systems targeting a variety of settings, ranging from the laboratory to a range of POC settings.

Experimental

Dry preservation of HRP conjugated antibody

A preservation formulation of HRP-conjugated anti-*Pf*HRP2 antibody (HRP-antibody) (Immunology Consultants Laboratory, Portland OR) at a concentration of 100 µg/ml was prepared in a mixture containing a range of (0-0.2 M) $FeSO_4$-EDTA (Fe-EDTA), 4% trehalose (Sigma Aldrich, Saint Louis, MO), 0.1% bovine serum albumin (BSA) (Sigma Aldrich, Saint Louis, MO) in PBS. The $FeSO_4$ was prepared in water and premixed with an equimolar EDTA solution to prevent precipitation in PBS. The mixture was filtered through a 0.2 µm pore size filter. Polystyrene microtiter well strips (Thermo Electron Corporation, Milford MA) were coated with 2 mg/ml BSA overnight, washed with water and dried. Glass fiber (grade 8964) (Ahlstrom, Helsinki, Finland) was laser cut (Universal Laser Systems, Scottsdale, AZ) to fit the wells of the polystyrene microtiter strip. The fluid capacity of each glass fiber pad was ~ 20 µl. Ten microliters of the HRP-antibody preservation mix was added to the glass fiber

pads and dried at 30 °C under vacuum (Genevac Inc, Gardiner NY) for 2 hours. The microstrips were then sealed in triplicate using a Foodsaver® vacuum sealing system. The samples were placed in a VaporLoc bag (LPS Industries, Moonache, NJ) with 2 grams of desiccant (Desiccare Inc, Pomona CA) per sample and stored at 45 °C for long-term storage.

Dry preservation of diaminobenzine

Diaminobenzidine (DAB) tablets (Sigma Aldrich, Saint Louis, MO) were dissolved in water in acidic conditions by adding HCl to pH 2.0 and then raising the pH to 7.0 with NaOH to achieve a concentration of 2 mg/ml. Trehalose at a concentration of 4% was added to the DAB solution, and the solution was filtered through a 0.2 µm pore size filter. Glass fiber pads were placed in a polystyrene microtiter well strip, and 20 µl of the DAB mixture were added to each sample. The samples were dried, packaged and stored at conditions similar to those described above for the HRP-antibody.

Colorimetric assay for HRP preservation

A colorimetric assay using the 3,3',5,5 tetramethylbenzidine (TMB) substrate (KPL Inc, Gaithersburg, MD) was used for testing the HRP activity. The glass fiber pads containing the dry HRP-antibody were rehydrated with 100 µl PBS to 10 µg/ml and vortexed for 1 minute. The samples were further diluted to a concentration of 200 ng/ml and 10 µl was pipetted into a microtitre plate. A control sample of fresh HRP-antibody was used for comparison. One hundred microliters of TMB containing hydrogen peroxide was added to the plate and immediately placed in a VersaMax ELISA microplate reader (Molecular Devices LLC, Sunnyvale, CA). Kinetics of the TMB oxidation by HRP to its blue-colored singly-oxidized product was measured at 650 nm every 15 seconds over a period of 5 minutes. The initial rate of the reaction was calculated by taking the first 8 readings, and the percent activity was calculated relative to the fresh HRP-antibody.

Lateral flow assay for HRP-antibody preservation

A dipstick-style lateral flow assay format was used for demonstrating the dry preservation of the HRP-antibody. A nitrocellulose membrane (Millipore, Billerica, MA) was cut using a CO_2 laser cutting system (Universal Laser Systems, Scottsdale, AZ) [31] into a "comb" structure consisting of multiple strips connected at the top. The assay used was a standard sandwich immunoassay for malarial antigen *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP2). A murine antibody to *Pf*HRP2 (Immunology Consultants Laboratory, Portland, OR) was patterned (Scienion, Berlin, Germany) at a concentration of 1 mg/ml onto the detection region of the nitrocellulose strip. A mock sample was created by adding 50 ng/ml of the recombinant malaria protein *Pf*HRP2 (40 kD) (ImmunoDiagnostics Inc., Woburn, MA) to fetal bovine serum (Invitrogen, Carlsbad, CA). The glass fiber pads containing the dried HRP-antibody conjugate were rehydrated to a concentration of 10 µg/ml in PBS pH 7.4 with Tween® 20 (PBST),

vortexed for one minute, and used as the label in the sandwich assay. Fresh DAB prepared in PBST to a working concentration of 0.125 mg/ml served as the substrate. Sodium percarbonate (Sigma Aldrich, Saint Louis, MO) at a concentration of 0.025% was added fresh to the DAB solution as the source of hydrogen peroxide (H_2O_2). A fresh sample of HRP-antibody conjugate was used as a control. The nitrocellulose comb with a cellulose wicking pad at one end was placed vertically into a 96-well microtiter plate containing 30 µl of antigen for 4 minutes. This was followed by two transfers into 30 µl PBST wash buffer for 4 min each. The comb was then transferred to wells containing HRP-antibody at 10 µg/ml for 4 min, followed by two more PBST washes. Finally, the comb was placed in 40 µl of DAB substrate for 6 minutes for the signal to develop at the detection zone. The assay membranes were then imaged wet using the procedure described below.

Lateral flow assay for DAB preservation

A dipstick format malarial assay, similar to the one used for the HRP preservation studies, was used to evaluate DAB preservation. Here, a fresh solution of secondary HRP-antibody conjugate at 10 µg/ml was used as the label. The lateral flow assay procedure was similar to the one described above. At different times of dry storage, samples of dry DAB were removed and rehydrated to a working concentration of 0.125 mg/ml with 40 µl of PBST containing the H_2O_2 source in the form of sodium percarbonate at a concentration of 0.025% and vortexed for one minute. The assay membranes were imaged as described below. The percent activity retained after dry storage of DAB was calculated relative to that of the fresh DAB.

Automated 2DPN ELISA card

As previously reported, the 2DPN assay card design [28] was adapted for performing on-card ELISA using the dry reagents. The device was a nitrocellulose (Millipore, Billerica, MA) assay membrane cut into a three-inlet network using a CO_2 laser cutting system. The *Pf*HRP2 antibody at a concentration of 1 mg/ml was patterned at the detection region of the membrane. The assay membrane along with a cellulose wicking pad (Millipore, Bellerica, MA) was housed on one side of a folding Mylar (Fraylock, San Carlos, CA) laminate material. The glass fiber pads with dry HRP-antibody and DAB substrate (stored dry for 2 weeks at 45 °C) and buffer pad were placed on the opposite side of the laminate housing such that when the device was closed, each pad would make contact with the appropriate inlet of the assay membrane. Ten microliters of a known concentration of *Pf*HRP2 antigen in FBS was added to the glass fiber pad containing HRP-antibody to rehydrate it to a concentration of 10 µg/ml. The dry DAB substrate pad was rehydrated with 40 µl of PBST containing hydrogen peroxide to give a concentration of 0.125 mg/ml. The buffer pad was filled with 80 µl of PBST. The assay card was folded

to simultaneously initiate flow of each reagent sequentially through the detection zone. The membranes were scanned after 20 minutes, and signal was quantified as decribed below.

Image capture and quantification

Assay membranes were imaged with a flatbed scanner (ScanMaker i900, MicroTek International, Inc., Cerritos, CA USA) in 48-bit RGB mode at a resolution of 600 dpi. The intensities in the region of interest (ROI) were quantified using ImageJ [32] by measuring mean green-channel intensity of each signal band and a background region within each strip. Each ROI was defined as a rectangular box around the detection line measuring 55 pixels x 10 pixels. The signal from a region 30 pixels upstream from the detection zone was used as the background signal intensity. The assay signal was calculated as the background-subtracted intensity normalized to the full intensity range of the image [33]. For each concentration, N=3 replicate measurements were performed. An estimate of the limit of detection (LOD) was obtained using LOD = (3σ)/S, where σ is the standard deviation of very low concentration measurements and S is the slope of the dose response curve in the linear region. In this case, the slope was taken over the lowest three concentrations and the standard deviation was calculated using the method of pooled variance (i.e. mean-centering each of the zero and lowest non-zero (6.25 ng/ml) concentration data sets and then calculating the standard deviation of the composite mean-centered data set). This procedure was motivated by the observation that the zero-concentration measurements had a much lower standard deviation than the lowest non-zero concentration measurements (this discrepancy may have been due to the modest number of replicates). In this case, the pooled variance may provide a more representative estimate of the LOD.

Results and discussion

Our goal in this study was to develop a method for the long-term dry preservation of the reagents used in ELISA (HRP-antibody and DAB substrate) and implement it in a lateral flow device. Glass fiber pads were chosen as a matrix for drying the reagents since they are convenient to handle during vacuum drying and provide a format appropriate for integration with our POC device.

We first tested the activity of the HRP enzyme in a colorimetric assay to determine the effects of long-term dry-storage at elevated temperature. We then tested the HRP-antibody function in a simple dipstick immunoassay using fresh DAB substrate to ensure that the preservatives did not interfere with the immunoassay. Next, we tested the stability of the dry-stored DAB substrate in the dipstick immunoassay using fresh HRP enzyme. Finally, we incorporated the dry reagents into an automated device and demonstrated ELISA in a simple card format.

Dry storage of enzyme (HRP) conjugated antibody

HRP conjugated to *Pf*HRP2 antibody (HRP-antibody) was dried on glass fiber pads in the presence of Fe-EDTA, trehalose, and BSA. We believe that the BSA in the preservation formulation could serve as a sacrificial protein coating of the surface of the glass fiber matrix during the drying process and reduce the loss of HRP-antibody adsorption to the matrix. The HRP activity was determined by measuring the conversion of TMB substrate to a blue product in a colorimetric assay. Kinetic measurements over a wide range of fresh HRP-antibody concentrations were performed in order to choose an HRP concentration in the linear measurement range for the assay (supplementary Figure S1); an HRP concentration 200 ng/ml was chosen for the kinetic measurements so that the assay would be responsive to decreases in enzyme performance.

First, a range of Fe-EDTA concentrations (0-0.2 M) was tested to determine the optimal Fe-EDTA concentration that provides enzyme preservation without interfering with signal generation or target labeling. The HRP activity was quantified by colorimetric assay, and the percent activities for fresh and dried HRP-antibody were calculated relative to the fresh HRP-antibody control with no preservatives. There was no effect of Fe-EDTA concentration on the HRP activity for freshly prepared samples (Figure 1a, gray bars). The optimum HRP activity in samples stored dry at 45 °C for 3 days was achieved using concentrations of 0.005-0.02 M Fe-EDTA. Below and above this range Fe-EDTA resulted in a decrease in enzyme activity (Figure 1a, white bars). A dipstick immunoassay using fresh DAB substrate was also performed to test for any interference of Fe-EDTA in the sandwich immunoassay. The dipstick assay results indicated that 0.005-0.02 M Fe-EDTA was the optimal range for enzyme stability (Figure 1b). Concentrations greater than 0.02 M Fe-EDTA in the dry formulation gave weaker signals using both fresh and dry HRP-antibody, which suggests interference with the sandwich immunoassay. Hence, 0.01 M Fe-EDTA was chosen for the study of long-term dry stability of the HRP-antibody at elevated temperature.

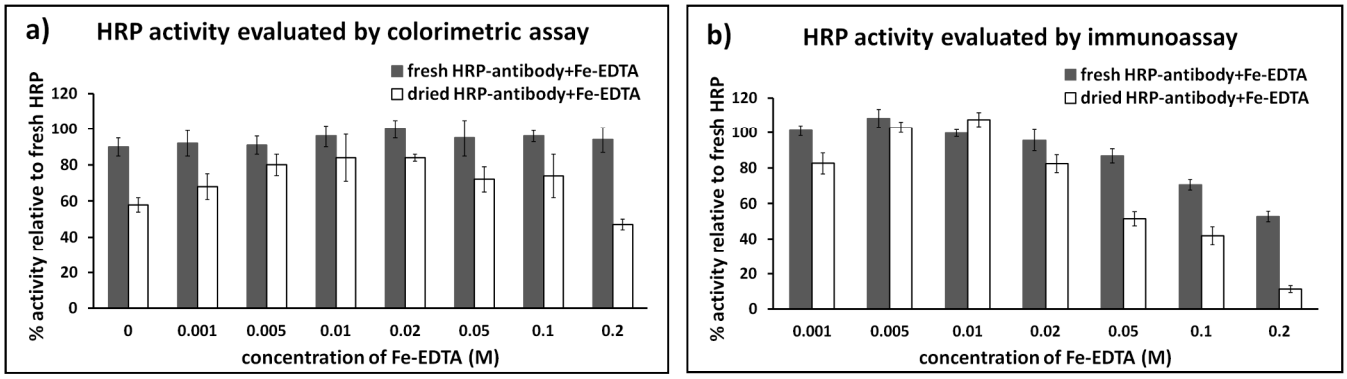


Figure 1. Tests to determine optimal concentration of preservatives for the HRP-antibody. Dry storage conditions were 3 days at 45 °C in presence of Fe-EDTA, 4% trehalose and 0.1% BSA. The fresh HRP condition tests for inhibition of HRP activity or immunoassay chemistry, and the dry HRP condition tests for additional effects on preservation of HRP-antibody performance. a) Plot showing the effect of a range of Fe-EDTA concentrations on the activity of HRP determined by a colorimetric assay. The percentage activity was calculated relative to the fresh HRP without Fe-EDTA and trehalose. The enzyme activity was maintained for a wide range of Fe-EDTA concentrations for both fresh and dry-stored HRP-antibody with optimum range between 0.005-0.02 M Fe-EDTA for the dry-stored HRP antibody. b) Plot showing the effect of a range of Fe-EDTA concentrations on performance of HRP-antibody measured in a full immunoassay. Note the consistency with data in a) for the best range. Further, concentrations greater than 0.01M Fe-EDTA in the dry stored HRP-antibody had an inhibitory effect on the sandwich assay.

Figure 2a shows the HRP activity for dry storage over 5 months at 45 °C. Enzyme activity was measured using TMB as the substrate relative to fresh HRP-antibody. The HRP-antibody dry-stored in the presence of Fe-EDTA, trehalose, and BSA retained 80% of the activity of the fresh control. In the presence of trehalose and BSA alone, the enzyme retained only 20% activity. The activity of HRP-antibody was also evaluated by a dipstick sandwich immunoassay using fresh DAB as the substrate (Fig. 2b). The HRP-antibody was functional with signal intensity similar to the fresh HRP-antibody.

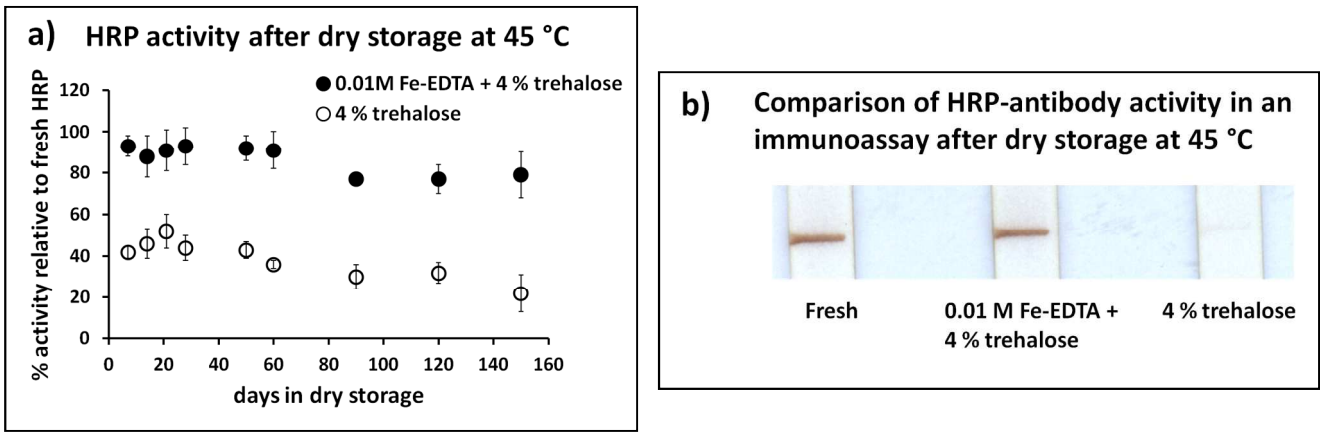


Figure 2. Activity of HRP-antibody after dry storage. a) Chart showing the HRP enzyme activity retained after dry storage at 45 °C at different time points as determined by colorimetry. The activity was compared to fresh reagent stored at 4 °C. The HRP enzyme in the presence of 0.01 M Fe-EDTA and 4% trehalose and 0.1% BSA retained ~ 80% activity after dry storage at 45 °C after 5 months. In the presence of 4% trehalose and 0.1% BSA the enzyme retained only 20% activity b) Images of a dipstick immunoassay using dry-stored (4 months, 45°C) HRP-antibody and fresh DAB substrate and H_2O_2 in a malarial sandwich assay. The signal is seen as a brown precipitate due to oxidation of DAB by HRP in presence of hydrogen peroxide. The HRP- antibody shows excellent functionality upon rehydration when stored dry in the presence of Fe-EDTA, trehalose, and BSA whereas trehalose alone showed significantly diminished activity.

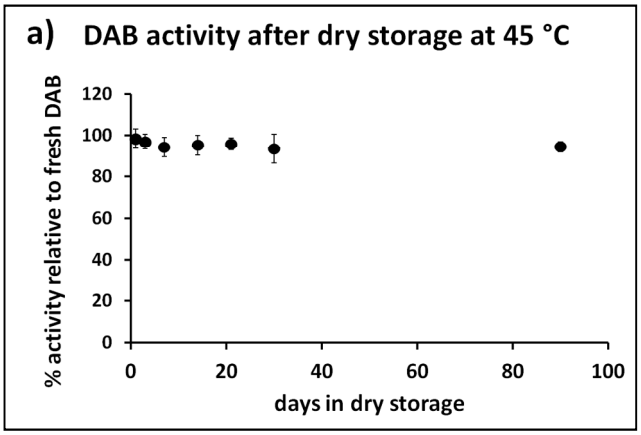
In our study, we found that adding $FeSO_4$ instead of Fe-EDTA complex reduced the enzyme activity to ~ 40% after 3 days of dry storage at 45 °C, which indicates that the Fe-EDTA complex had a role in the preservation. Also, the $FeSO_4$ precipitated in the PBS buffer and adding equimolar concentration of

EDTA prevented precipitation. Fe-EDTA alone without trehalose reduced the enzyme activity to <20% after one day of dry storage at 45 °C. Also EDTA alone in presence of trehalose gave results similar to trehalose alone. In addition, the HRP sample stored in trehalose, which gave diminished activity, was tested by adding Fe-EDTA after rehydration. This experiment did not improve the HRP activity. In summary, the Fe-EDTA complex in the presence of trehalose had a stabilizing effect on HRP during dry storage at elevated temperature.

Dry preservation of substrate diaminobenzidine

Diaminobenzidine (DAB) has been a commonly-used substrate for HRP, especially in the field of immunocytochemistry. Advantages of DAB includes that it dissolves easily in aqueous media and the oxidized product self-precipitates; in the context of our assay this means that DAB deposits as a stationary signal at the detection line of the paper strip. In contrast, precipitation of the oxidized TMB product formed in ELISA requires addition of propriety precipitating components.

DAB substrate was dried in glass fiber pads in the presence of trehalose and stored at 45 °C. The stability of DAB was evaluated using a dipstick immunoassay over a timescale of 3 months. First, the dipstick malarial assay using fresh reagents was used to determine the linear range for signal generation using DAB for testing its dry storage stability (such that loss in DAB activity would be detectable). The linear range of the assay was at concentrations lower than 0.15 mg/ml, and thus a concentration of 0.125 mg/ml of DAB was chosen for the dry storage study (Supplementary Figure S2a). One of the critical components in the enzyme-based immunoassay is H_2O_2, which is essential for oxidation of the substrate by HRP. For all the experiments in this study, we used sodium percarbonate, which generates H_2O_2 when it is dissolved in aqueous buffer. A concentration of 0.025% sodium percarbonate was chosen based on a study of the effect on H_2O_2 concentration on signal intensity in a dipstick assay (Supplementary Figure S2b).



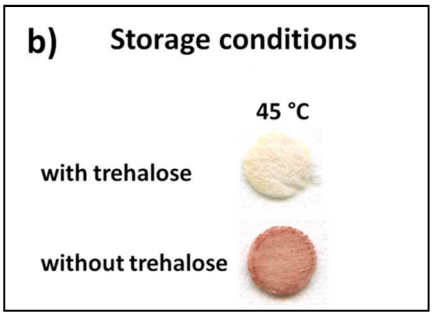


Figure 3. a) Plot showing percent DAB activity retained after dry storage as determined by a dipstick immunoassay. DAB substrate stored dry at 45 °C in the presence of 4% trehalose in a glass fiber pad was tested periodically for its functionality using fresh secondary HRP- antibody. The DAB substrate retained full functionality upon rehydration after 3 months of dry storage when compared to fresh DAB. b) Samples of glass fiber pads containing dry DAB stored for one month with and without trehalose. Trehalose suppressed auto-oxidation of DAB during dry storage.

Long-term stability study of DAB stored at 45 °C was tested in a dipstick immunoassay using fresh HRP-antibody. Figure 3 shows that DAB was preserved with >90% activity for at least three months at 45 °C (Fig 3a). We observed that adding trehalose had a suppressive effect on the auto-oxidation of DAB during dry storage compared to samples stored without trehalose (Fig 3b). It was also necessary to store DAB in the dark to prevent photooxidation.

Automated ELISA in 2DPN device

A POC device with on-board dry reagents for enzymatic signal amplification could provide high sensitivity detection for low-resource settings. A previously reported 2DPN device [28] was adapted for demonstration of ELISA for the POC.

Figure 4a shows a folding 2DPN device with integrated dry reagent pads that performs an automated ELISA for a malarial biomarker (*Pf*HRP antigen) with a single user activation step. Antigen-spiked sample was added to a pad containing dry HRP-antibody, PBST buffer containing sodium percarbonate was added to a pad containing dry DAB substrate, and PBST buffer was added to a buffer pad as a wash fluid. While on-device storage of sodium percarbonate was not done in this study, it is sold in a dry form that is stable at room temperature. The sodium percarbonate could be stored as a powder in the 2DPN device at a convenient location, and would generate H_2O_2 upon dissolution.

After two minutes to allow for reagent rehydration, the card was folded to initiate the sequential delivery of reagents through the detection zone. The antigen-antibody complex with the HRP-antibody label moved through the first inlet and across the detection zone, followed by the DAB substrate plus hydrogen peroxide from the second inlet, and finally a wash buffer from the third inlet. Figure 4b shows an example result from an automated ELISA card. The signal from the DAB precipitate can be easily visualized by eye at the detection zone. This method of on-card enzyme signal amplification can also be quantified using a webcam or a flat-bed scanner. Figure 4c shows images for an antigen dilution series and the corresponding dose response curve (Figure 4d). The LOD using the 2DPN card for the malarial antigen was 6.5 ng/ml. The LOD is comparable to laboratory ELISA for *Pf*HRP2 of 4 ng/ml [34]. The *Pf*HRP2 levels in clinical blood samples can range from as low as 10 ng/ml to as high as 10^3 ng/ml [35]. Apart from diagnosing an active infection, the 2DPN card could also be used in the malaria elimination

programs which aim to reduce parasite transmission in the population where higher sensitivities are required (P. LaBarre, personal communications).

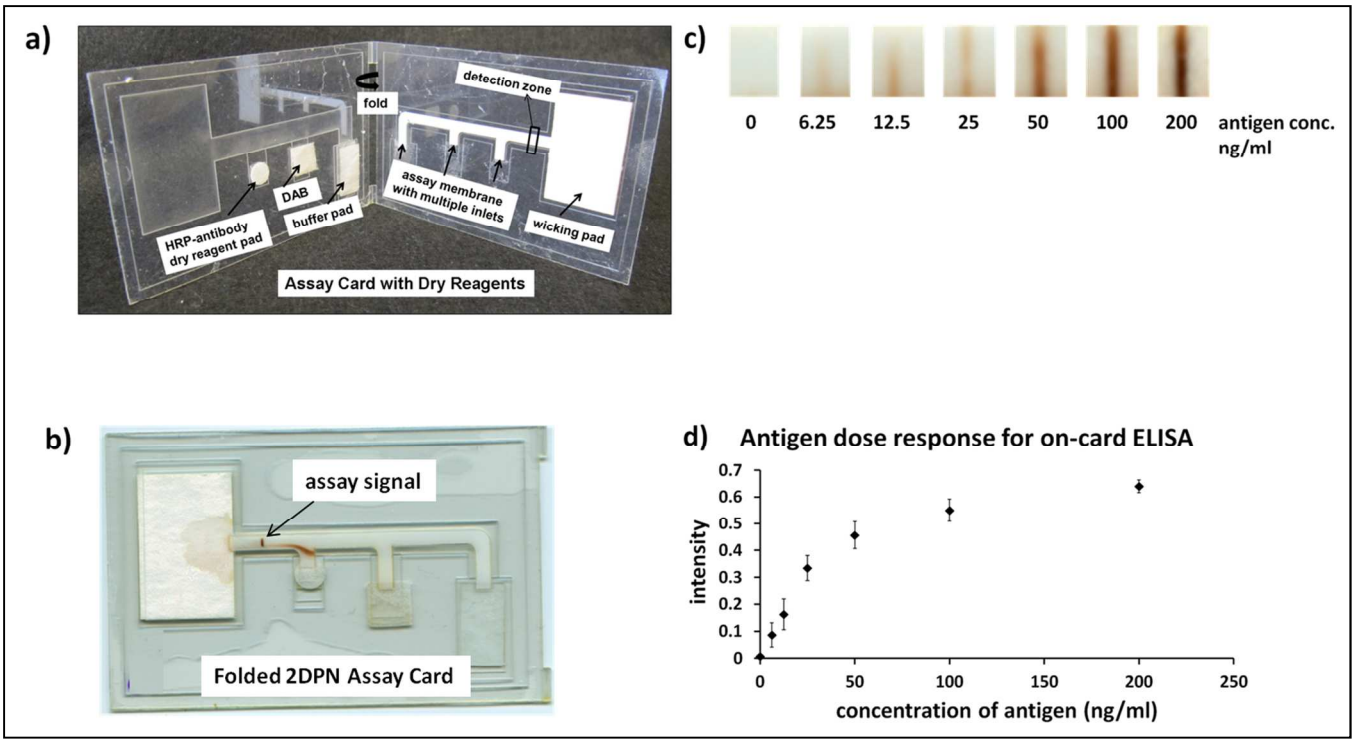


Figure 4. 2DPN device with incorporated dry reagents for automated ELISA. a) Folding 2DPN device showing assay membrane on one side with three inlets for sequential reagent delivery, and glass fiber pads containing dry- preserved HRP labeled secondary antibody, DAB and buffer on the other side, assembled on a Mylar laminate material. Malarial antigen spiked in fetal bovine serum and buffer containing hydrogen peroxide is added to rehydrate the dry HRP-antibody and DAB respectively. The device is folded to simultaneously activate the reagent flow through the device. b) Folded device showing malarial sandwich immunoassay signal development. A streak of DAB precipitate is seen near the first inlet. This is due to the two reagents (HRP-antibody and DAB substrate) flowing back to back. This however did not have a negative impact on the assay. Streaking could be eliminated by adding a wash step between the HRP-antibody and DAB delivery. c) Images of the detection line for varying antigen concentration. d) Dose response curve for the malarial antigen using 2DPN device with dry reagents.

Conclusion

We reported a method for dry preservation of reagents used in ELISA. We showed that an HRP-antibody conjugate and its substrate DAB retained a high level of activity after long-term dry storage at 45°C. Our method for drying the reagents onto a glass fiber pad has the benefit of easy incorporation into POC devices, including conventional microfluidic [36] or paper-based devices [9, 28]. We used a simple vacuum-drying method over the time-consuming, cumbersome, and expensive lyophilization method.

This method can be easily adapted for manufacturing in low-resource settings. Further, we have demonstrated an automated ELISA using a 2DPN device with dry reagents integrated into it. Future work will include adapting the device to accept a whole blood sample through use of a plasma extraction membrane that would separate the blood cells [36, 37]. In addition, we are also working on paper-fluidic valving tools to demonstrate the automated process of fluid delivery and metering in the 2DPN device [38]. Dry preservation of enzyme-based signal amplification reagents could be used for a variety of applications and has particular advantages for use in POC devices for enhanced sensitivity, portability, and ease-of-use in low resource settings.

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