



Polymerization-based Signal Amplification for Paper-Based Immunoassays

Citation

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Paper-based Diagnostic Device with Polymerization-based Signal Amplification

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Diagnostic tests in resource-limited settings (RLS) require technologies that are affordable and easy to use with minimal infrastructure. Colorimetric detection methods that produce results that are readable by eye, without reliance on specialized and expensive equipment, have great utility in these settings. Existing colorimetric methods based on enzymatic reactions or gold nanoparticles (AuNP) produce results that must be read within a specified time interval to ensure their validity. In some instances, the user incorrectly interprets the result because the color that is produced is faint. We have developed a colorimetric method that integrates a paper-based immunoassay with a rapid, visible-light induced polymerization to provide high visual contrast between a positive and a negative result. Using Plasmodium falciparum histidine-rich protein 2 (PfHRP2) as an example, we have demonstrated that this method allows visual detection of proteins in complex matrices such as human serum. The method we describe provides quantitative information regarding analyte levels when combined with cellphone-based imaging. It also allows the user to decouple the capture of analyte from signal amplification and visualization steps. We believe that this added flexibility to decouple steps in the assay has the potential to decrease incidences of false readouts due to timing errors in busy point-of-care (POC) settings.

Analytical devices made of cellulosic materials (e.g. filter and chromatography paper) are attractive for RLS because of their low cost,^[1-4] ease of fabrication,^[3-5] and porous structure that facilitates capillary flow. 2D and 3D microfluidic paper-based analytical devices (μ PADs) have been developed and used for detection of small molecules,^[2,6] metals,^[7,8] and proteins^[1,3,9]. A crucial component of an analytical test is the visualization mechanism that allows users to determine the results of a test. A wide range of visualization mechanisms are available for use with paper surfaces,^[10] but POC tests in RLS are constrained by cost and inadequate infrastructure. Colorimetric methods based on enzymatic reactions or gold nanoparticles (AuNP) are, therefore, widely used in paper-based POC tests because they provide an equipment-free readout.^[10-13] These methods sometimes produce non-uniform color changes^[14] and low visual contrast between a positive and a negative result^[9] that lead to subjectivity in interpretation of the result by the user. This subjective interpretation decreases the accuracy and sensitivity of this kind of test. Color development after capture of the analyte from a patient sample

using a bioactive paper surface takes anywhere from 10 minutes for enzymatic reactions^[2] to 20-30 minutes for AuNP-based sensing with^[9] or without^[12] silver enhancement. The colorimetric results are time-sensitive, leading to a false negative test if read prematurely and a false positive test if read after a specified time.^[15] The motivation for this work was to develop a polymerization-based colorimetric sensing method for use with paper immunoassays to address these limitations of existing colorimetric methods.

A polymerization response can be coupled to detection of an analyte using a method termed polymerization-based amplification (PBA)^[16-21]. In PBA, photoinitiators are localized to regions where specific molecular binding events have occurred through covalent coupling of the photoinitiator to one of the affinity reagents used in the assay. When the photoinitiator molecules are supplied with an appropriate dose of light in the presence of acrylate monomers, they initiate a free-radical polymerization reaction to generate an interfacial hydrogel. The result of the polymerization process is the formation of a hydrogel only in areas where the local concentration of the photoinitiator near a binding surface is sufficient to overcome competing inhibition reactions and initiate polymerization. PBA is rapid with a reaction time of less than 100 seconds and can be performed in air, without the need for oxygen removal via purging,^[21] by using an eosin/tertiary amine initiation system that can overcome oxygen inhibition through an eosin regeneration mechanism.^[22] The reactants and key elementary reactions in the overall polymerization reaction initiated by eosin and a tertiary amine co-initiator are described in Supplementary Information, Discussion Section 1. PBA provides the flexibility of choosing the start and end point of the amplification process by turning the light source on and off, respectively. We hypothesize that the ability to control the start and end of a rapid amplification process could be useful in avoiding false negative and false positive results that arise from timing errors.

PBA was previously developed using bioactive glass surfaces and the colorless hydrogel was swollen with a concentrated dye solution to aid visualization by eye.^[18,20,21] However, the swelling method could not be used with paper because dyes adhere non-specifically to paper. This non-specific adhesion led to low contrast between the hydrogel and the background and generated false positive results (Supplementary Information,

Discussion Section 1). In this work, we have adapted PBA to detect molecular binding events on a paper surface. We developed a new visualization method for PBA by using the pH-dependent color change of a pH indicator to detect the formation of the hydrogel. We have successfully used this polymerization method to generate colorimetric results, easily perceptible to the unaided eye, for the immuno-detection of *Pf*HRP2 using modified chromatography paper. *Pf*HRP2 is a soluble protein released into the blood stream during infection by *Plasmodium falciparum*.^[23] It is a well-established biomarker for falciparum infection and several commercial diagnostic tests are based on its detection.^[24]

To integrate PBA with an immunoassay for detection of *Pf*HRP2 (Scheme 1), we prepared chromatography paper for covalent immobilization of antibodies by oxidation of a fraction of the hydroxyl groups in cellulose to aldehyde groups (Supplementary Information, Figure S1).^[25,26] The surfaces were printed with wax and treated with heat to create hydrophilic test zones that are separated by hydrophobic wax barriers^[27]. A monoclonal capture antibody specific to *Pf*HRP2 (Arista Biologicals, Clone 44) was covalently coupled to the surface by reaction of aldehyde groups on the paper with the primary amine groups of the lysine residues of the antibody. When this surface containing the *Pf*HRP2-specific capture antibody was contacted with a solution containing *Pf*HRP2, the capture antibody formed a complex with *Pf*HRP2, resulting in a positive test surface (Figure 1). In order to associate a polymerization response with the presence of *Pf*HRP2 on the surface, we coupled eosin, a photoinitiator, to a monoclonal reporter antibody specific to *Pf*HRP2 (Arista Biologicals, Clone 45). The eosin-modified reporter antibody (containing an average of 7 eosin molecules per antibody molecule) was prepared by reaction of the isothiocyanate group of eosin 5-isothiocyanate with the amine groups of the antibody's lysine residues (Supplementary Information, Figure S2). When a paper surface containing the capture antibody-*Pf*HRP2 complex was contacted with the eosin-modified reporter antibody, the reporter bound to *Pf*HRP2. This binding step localized eosin on the surface where *Pf*HRP2 had been captured. In contrast, for a negative test surface, eosin was not localized if *Pf*HRP2 was not present to bridge the two antibodies. We placed positive and negative test surfaces prepared in this way in contact with a 20 μ L drop of an aqueous amplification solution and illuminated them from above

using an array of light-emitting diodes (LEDs) ($\lambda=532\text{nm}$, 30 mW/cm^2). The amplification solution contained 150mM triethanolamine (TEA), 200mM (poly)ethyleneglycol diacrylate (PEGDA), 100mM 1-vinyl-2-pyrrolidinone (VP), 1.6mM phenolphthalein, and $0.35\text{ }\mu\text{M}$ free eosin. The pH of the solution was adjusted to 7.9 using hydrochloric acid (HCl). The photoinitiation time was chosen as 90 seconds so that the photoinitiator density was sufficient to overcome oxygen inhibition and initiate polymerization on positive test surfaces but not on the negative test surfaces. As the hydrogel formed on the positive test surfaces, phenolphthalein that was present in the aqueous solution became physically trapped in the cross-linked network. The hydrogel remained on the paper surface even after the surface was washed to remove unreacted amplification solution.^[17,28] The hydrogel that formed on the surface after polymerization was transparent. However, when $2\text{ }\mu\text{L}$ of 0.5M NaOH was added to the surface, the pH increased above 8 and phenolphthalein changed from colorless to bright pink. The change in color occurred immediately upon the addition of NaOH (Supplementary Information, Discussion Section 2) and allowed visual detection of the hydrogel by the unaided eye. In contrast, no hydrogel formed on the negative test surfaces, which means that the residual phenolphthalein was washed away with the unreacted monomer and no change in color was observed upon addition of $2\text{ }\mu\text{L}$ of 0.5M NaOH to these surfaces. The pH-dependent hydrogel visualization thus allowed us to clearly differentiate between a positive and a negative test surface based on a colorimetric response.

Each component of the amplification solution used to detect molecular recognition at a paper surface serves a unique purpose. TEA acts as co-initiator; the multifunctional monomer PEGDA is needed to form a crosslinked network; VP is a fast diffusing, low molecular weight monomer for improved kinetics; free eosin is used to overcome oxygen inhibition^[21,22]; phenolphthalein and HCl are included for visualization. Phenolphthalein, a weak acid, is colorless at a pH less than 8. As pH increases above 8, the equilibrium shifts in favor of the dianionic form that is pink in color (Supplementary Information, Figure S3). The amplification solution without HCl is basic with a pH of 9.3 because of the presence of TEA. If phenolphthalein is added to the monomer formulation without pH adjustment, it is predominantly present in its dianionic state that strongly absorbs at the same wavelength as eosin and competes with eosin for absorption of light during the

initiation step. Therefore, the pH of the solution was adjusted to 7.9 so that phenolphthalein was present in its acidic, colorless form. We confirmed the state (acidic/dianionic) of phenolphthalein in the amplification solution before and after adjustment of the pH using UV-visible spectroscopy (Supplementary Information, Figure S4). The concentration of phenolphthalein added to the solution was maximized within the limits of solubility to provide an intense colorimetric response.

We optimized the paper-based immunoassay using *Pf*HRP2 solutions prepared in 1% PBSA (1%w/v bovine serum albumin (BSA) in phosphate-buffered saline (PBS) solution). The impact of variables such as the oxidation of the surface of the paper, the concentrations of capture and reporter antibodies and the incubation times for capture antibody, *Pf*HRP2 and reporter antibody on the amount of surface-localized eosin was determined experimentally using fluorescence measurements (Supplementary Information, Figures S5, S6, S7 and S8).

In order to determine the limit-of-detection (LoD) of *Pf*HRP2 using the immunoassay format described in Figure 1 with polymerization-based signal amplification, we tested nine different concentrations of *Pf*HRP2 in 1% PBSA ranging over two orders of magnitude, 130nM to 1.3nM. The negative surfaces were contacted with 1% PBSA without any *Pf*HRP2. All surfaces were illuminated for 90 seconds and visualized using NaOH. The surfaces were imaged using a cellphone camera immediately after the addition of NaOH. We used ImageJ to quantify the average intensity of the colorimetric response. In brief, color images were split into red, green and blue channels. The pink polymerization response appeared as a dark area in the green channel but did not appear in the blue channel (Supplementary Information, Table S1). Therefore, for each result, the colorimetric intensity of the polymerization response was quantified by subtracting the average intensity of the test zone in the green channel from the average intensity of the test zone in the blue channel. The resulting number represents the reduction in the light intensity due to the presence of phenolphthalein trapped within the interfacial hydrogel. We used two approaches to define LoD of *Pf*HRP2, i) visual LoD, defined as the concentration where all the replicates show a visible colorimetric response, and ii) calculated LoD, defined as the minimum concentration that gives an average colorimetric signal that is higher than the average signal from the negative surface by three times the

standard deviation of the mean from the negative surface. Representative images from one of five independent dose-response trials are shown in Figure 2A with three replicates at each concentration of *Pf*HRP2. The visual LoD was 7.2nM because all replicates (n=19) with a *Pf*HRP2 concentration of 7.2nM and higher showed a bright pink hydrogel. All replicates with *Pf*HRP2 concentrations of 2.3nM, 1.3nM, or 0nM (negative surfaces) did not have a visible hydrogel. At a *Pf*HRP2 concentration of 4.1nM, 9 out of a total of 19 replicates from five different trials showed a visible hydrogel. This response is consistent with the threshold nature of PBA^[16,20] near the limit of detection, where small differences in the number of bound proteins can push the initiator density either slightly above or slightly below the concentration threshold where propagation reactions become competitive with inhibition reactions.^[29] The colorimetric intensity data (Figure 2B) were also fitted to a sigmoidal curve using a non-linear regression model (see Supplementary Information) and the calculated LoD was 5.8 nM according to the definition given above. The close agreement between the visual and the calculated LoD indicates that if a hydrogel is present on the paper surface, it can be reliably detected by the unaided eye without any need for image capture and image processing. Therefore, for qualitative assays where the exact concentration of analyte is not needed for making clinical decisions, PBA can be a useful tool for unambiguous visual detection. At the same time, if quantitative information about an analyte is required, paper-based PBA can be used in conjunction with mobile-based image capture and image processing. We observed that uniform lighting during image capture was important for reproducibility in image quantification for images taken at different times of the day. Different lighting at the time of image capture resulted in different colorimetric intensity values for the same concentrations of *Pf*HRP2. Using an ordinary desk lamp to illuminate the paper surface during image capture solved the problem of non-uniform light and gave reproducible colorimetric intensity values (Supplementary Information, Discussion Section 3).

We evaluated the effect of a complex matrix such as serum on the performance of the assay. In brief, we added 1μL of 64 μM (5mg/mL) stock solution of *Pf*HRP2 in 2% PBSA to 499μL of human serum to make a 130nM solution. The 130nM solution was serially diluted with serum to a concentration of 1.3nM. Undiluted human serum, without any added *Pf*HRP2, was used for the negative surfaces. A polymerization time of 50

seconds (See Supplementary Information, Discussion Section 4) was used to differentiate between positive and negative responses for this assay. All the other steps were identical to the assay carried out in PBSA. Figure 3 shows that the visual LoD of *Pf*HRP2 in human serum is similar to the visual LoD obtained in PBSA. The mean concentration of plasma *Pf*HRP2 has been reported as 28nM in a cohort of 337 adult patients with falciparum malaria.^[30] This concentration falls well within the dynamic range of the polymerization-based colorimetric method that we have developed.

In addition to conducting several independent trials with several replicates for each data point in each trial, we further verified the robustness of the assay by testing the stability of the photoinitiator and the stability of the hydrogel on the surface. We performed two different storage experiments. In a first experiment, we prepared surfaces containing covalently coupled anti-*Pf*HRP2 capture antibody, incubated them with either 130nM *Pf*HRP2 in 1% PBSA (positive) or with 1% PBSA without any *Pf*HRP2 (negative) and then contacted them with eosin-modified reporter antibody. These test surfaces were stored in air, at room temperature in a closed drawer for 1, 3, 7, 14, and 21 days before the polymerization was performed. The surfaces were illuminated for 90 seconds and visualized immediately using NaOH. Figure 4A shows representative images of the surfaces stored before polymerization. For comparison, positive and negative surfaces that were not stored (day 0) are also shown. The colorimetric readout is similar despite the difference in storage duration indicating that eosin is stable on the surface and can initiate the polymerization process after storage for extended periods of time.

In a second experiment, the paper surfaces were prepared as for figure 4A, then contacted with amplification solution and illuminated for 90 seconds immediately after treatment with eosin-modified reporter antibody. The surfaces were washed to remove the unbound monomer and stored in a drawer for 1, 3, 7, 14 and 21 days before NaOH was added for visualization. Figure 4B shows representative images of the surfaces stored after polymerization. For comparison, positive and negative surfaces that were not stored (day 0) are also shown. The hydrogel resulting from the polymerization process can be rehydrated with NaOH solution after up to three weeks in storage to generate a strong colorimetric response, indicating that phenolphthalein remains functional and trapped in the hydrogel during storage.

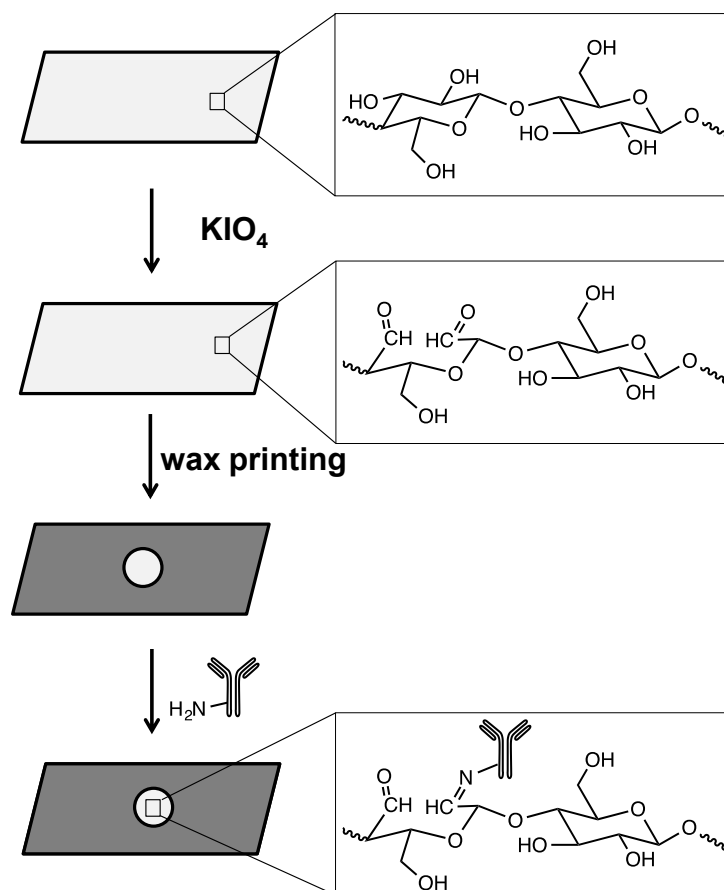
The above storage experiments demonstrate that using PBA with paper devices provides two separate points where the assay can be stopped, stored and restarted. This ability adds flexibility to the assay and has the potential to minimize false readouts due to time constraints in situations where only a few health workers are tending to the needs of many patients. It can also eliminate the need for sample collection and storage because it creates the possibility of self-testing by patients at home followed by polymerization and diagnosis later in-person or after sending the test to a health facility (for example, by mail).

We have described a new pH-responsive polymerization-based colorimetric sensing method for use with paper immunoassays that i) is rapid; it takes less than 100 seconds for the polymerization reaction after capture of analyte and color development is instantaneous upon addition of NaOH ii) generates results that are easily-perceptible using the unaided eye, iii) provides the flexibility to store the assay and control the start of the polymerization and the visualization steps, and iv) provides quantitative information when combined with cellphone-based image capture and image processing. We have used a sandwich immunoassay for *Pf*HRP2 detection as a model, but this method can be easily adapted for all types of immunoassays (direct or indirect) where a reporter antibody is used because the photoinitiator can be covalently attached to the lysine residues of any antibody or protein molecule to couple the polymerization response to detection of a specific analyte. The above advantages of PBA on paper come at the cost of requiring a source of light for illumination. We used a portable, electricity-powered LED light source in the laboratory. However, we can easily envision a portable, battery-powered LED device that can be adapted for use in RLS. Because the focus of the work was on development of the polymerization method, rather than building a complete device, we used a flow-through system where a user performs wash steps and adds the next solution. Strategies that have been used successfully to reduce the number of steps performed by users such as implementation of assays in a 2D paper network^[31] or a paper-based 3D microfluidic format^[32] could be used to reduce the number of steps here as well. Though the new method is not ready for field-testing yet, it addresses key limitations of existing colorimetric methods on paper. We expect that the advantages

provided by PBA-based colorimetric sensing hold promise for reducing inaccurate reading of results in rapid screening tests in field settings.

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Scheme 1: Preparation of paper to enable detection of *Pf*HRP2. A fraction of the hydroxyl groups of cellulose were oxidized to aldehyde groups by reaction with potassium (meta)periodate. The modified paper was printed with wax and heated to create hydrophilic test zones surrounded by hydrophobic wax barriers. An antibody that captures *Pf*HRP2 was covalently attached to the paper surface through a Schiff base linkage between aldehyde groups on the paper and the amine groups of the lysine residues of the antibody.

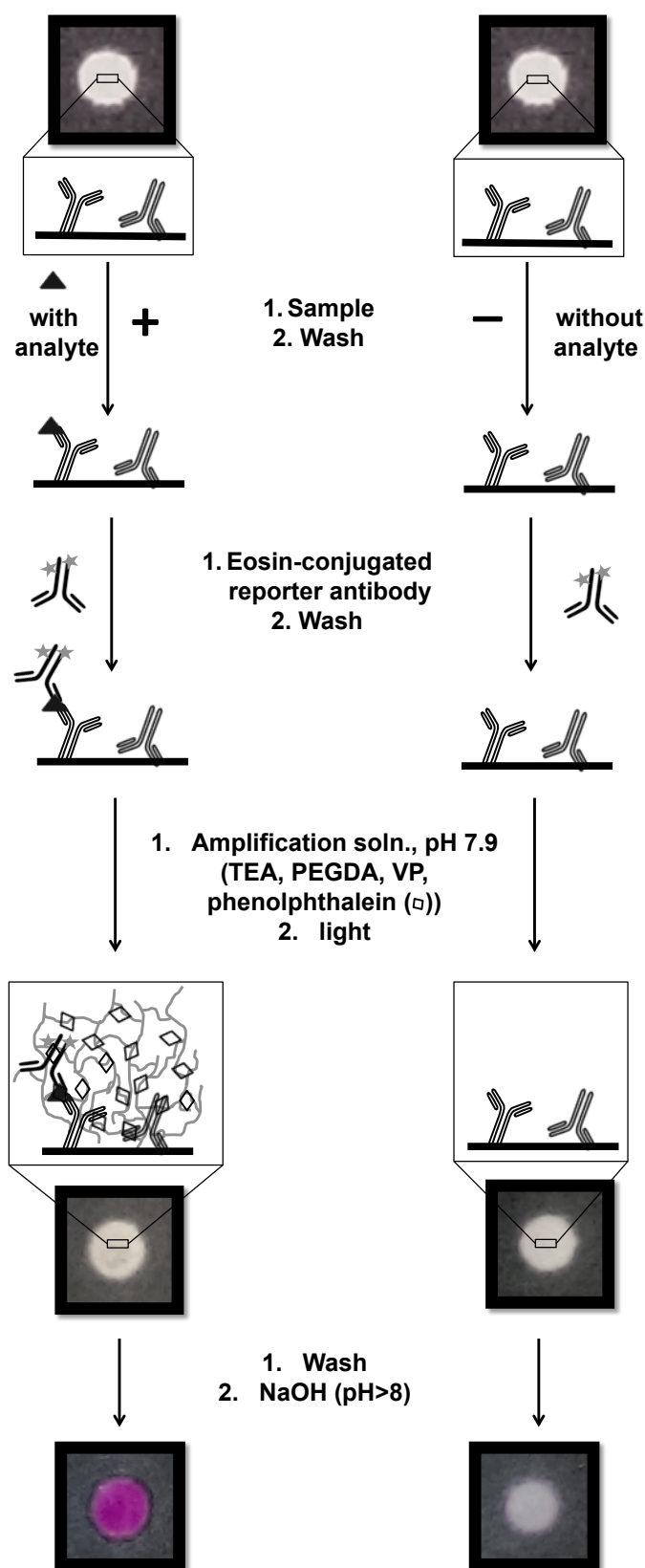
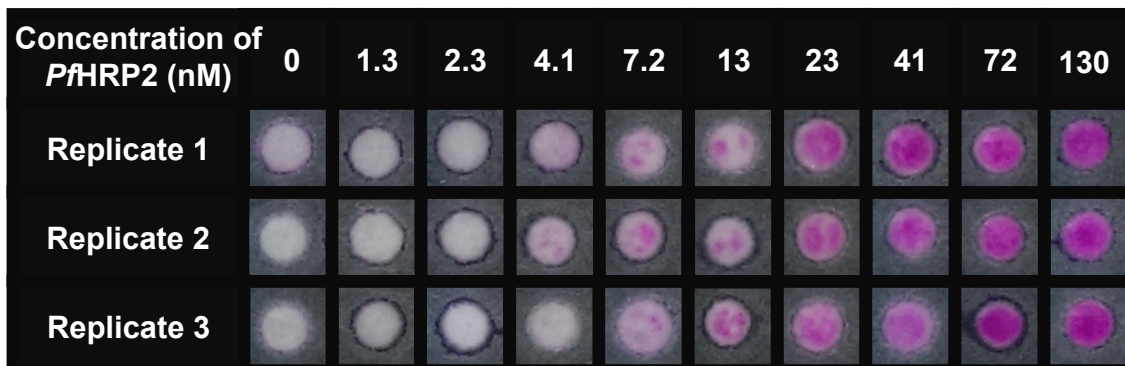


Figure 1: Using a paper immunoassay and polymerization-based amplification to detect *PfHRP2*. Paper surfaces described in Scheme 1 were contacted with a buffered solution containing *PfHRP2* (+) and a buffered solution without *PfHRP2* (-). After washing to remove the excess sample, the surfaces were contacted with an eosin-conjugated reporter antibody. After washing to remove the unbound reporter antibody, the surfaces were contacted with an aqueous amplification solution containing poly(ethylene glycol) diacrylate (PEGDA), triethanolamine (TEA), 1-vinyl-2-pyrrolidinone (VP) and phenolphthalein and irradiated. The surfaces were washed to remove unreacted solution and contacted with 2 μ L of 0.5 M NaOH. The positive test surface became bright pink and the negative test surface remained white.

(A) Detection of *Pf*HRP2 in buffer



(B) Quantification of the intensity of the polymerization response

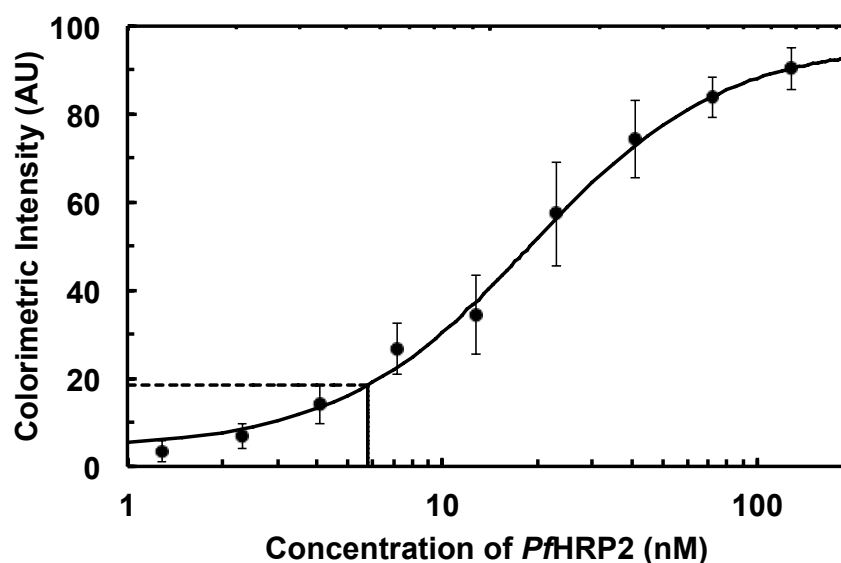


Figure 2: Detection of *Pf*HRP2 (0-130 nM) in a buffered solution by using polymerization-based amplification on paper. (A) Representative images of the colorimetric results for detection of various concentrations of *Pf*HRP2. The visual LoD, the minimum concentration at which all replicates give a positive polymerization response, was determined to be 7.2 nM. (B) Quantitation of the intensity of the colorimetric results for detection of *Pf*HRP2. Each data point is an average of eight replicates and error bars indicate standard deviation. The data were fitted to a sigmoidal curve and the calculated LoD, the concentration of *Pf*HRP2 that produces an average signal that is higher than the average signal from the negative control (0 nM *Pf*HRP2) by three times the standard deviation of the mean signal from the control, was 5.8 nM. The vertical dashed line indicates the calculated LoD of *Pf*HRP2 and the horizontal dashed line indicates the colorimetric intensity at the calculated LoD.

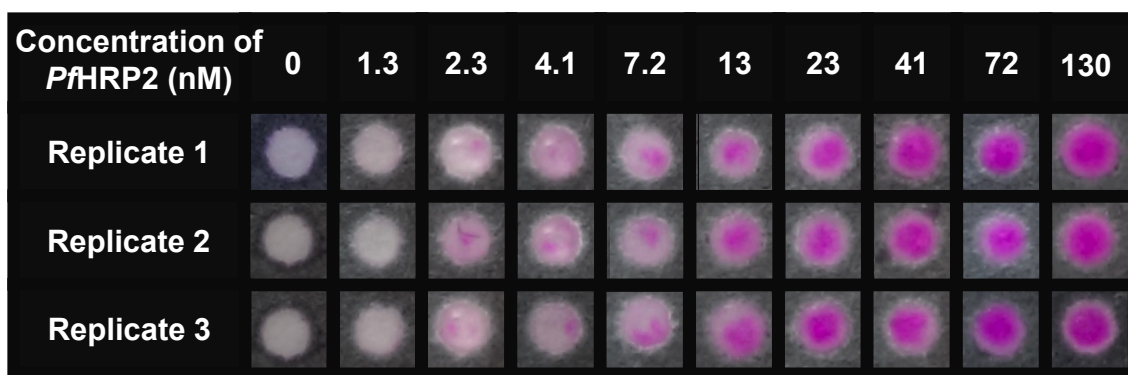
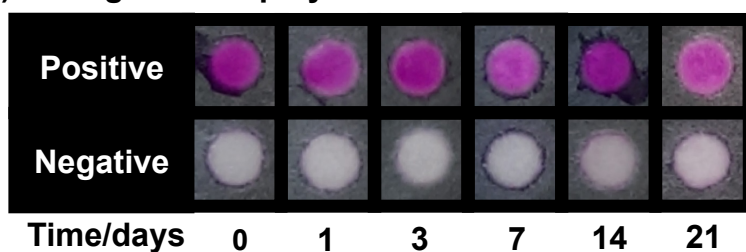


Figure 3: Colorimetric detection of *Pf*HRP2 (0-130 nM) in human serum using paper-based PBA.

(A) Storage before polymerization



(B) Storage after polymerization

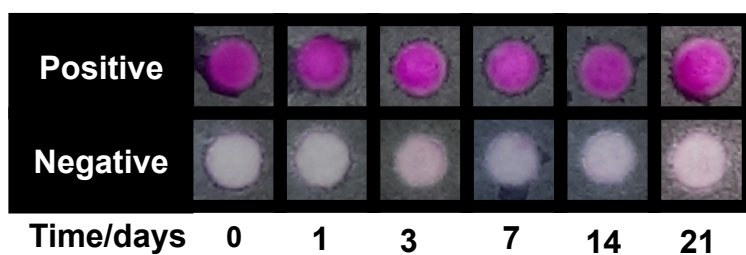


Figure 4: Colorimetric detection of positive (130 nM *Pf*HRP2) and negative (0 nM *Pf*HRP2) surfaces stored in air, at room temperature, in a closed drawer for 0-21 days (A) before polymerization, and (B) after polymerization but before visualization.

Supplementary Information

Paper-based Diagnostic Device with Polymerization-based Signal Amplification

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Materials and methods

Materials

Whatman No. 1 chromatography paper, lyophilized bovine serum albumin (BSA) and glycerol were purchased from VWR (Radnor, PA, USA). Gel blot paper (GB003, 15 cm × 20 cm) was obtained from Whatman, Inc. (Sanford, ME, USA). Potassium periodate, poly(ethylene glycol) diacrylate ($M_n=575$) (PEGDA), triethanolamine (TEA), 1-vinyl-2-pyrrolidinone (VP), eosin Y disodium salt, 2,4-dinitrophenylhydrazine, 10X phosphate buffered saline (PBS), phenolphthalein, Tween® 20 and sterile-filtered US-origin human serum (from human male AB plasma) were purchased from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. Eosin 5-isothiocyanate (EITC) was purchased from Marker Gene Technology (Eugene, OR, USA). Tris(hydroxymethyl)aminomethane (Tris) and sodium chloride were purchased via VWR from Avantor Performance Materials (Center Valley, PA, USA). Lyophilized *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2) was purchased from CTK Biotech (San Diego, CA, USA). The anti-*Pf*HRP2 IgG monoclonal antibodies (capture and reporter) were purchased from Arista Biologicals Inc. (Allentown, PA, USA). ABMAL-0444 (Clone 44) was used as the capture antibody and ABMAL-0445 (Clone 45) was used as the reporter antibody. UltraCruz™ Micro G-25 Spin Columns were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and PD-10 Desalting Columns were purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA).

Preparation of aldehyde-functionalized paper

Paper with aldehyde functional groups was prepared by soaking sheets (3'' × 8'') of Whatman No. 1 chromatography paper in a 0.03 M KIO₄ solution at 65 °C for 2 hours.^[1] After the reaction, the sheets were washed three times by dipping them in fresh deionized water (diH₂O) for one minute each and pouring off the water at the end. After the last wash, the sheets were blotted with paper towels and dried in a desiccator for at least 12 hours. Each dry sheet was taped to a regular 8'' × 11'' A4 printing paper and a wax mask containing circular wax-free regions (3 mm in diameter) was printed on them using a solid ink printer set to the default parameters for photo-quality printing. The A4 paper

was removed and the printed sheets were placed in an oven (150 °C) for 90 seconds. As a result of the heat, the wax melted and spread through the thickness of the paper^[2] and created circular (2 mm in diameter) hydrophilic test zones separated by hydrophobic wax barriers. The hydrophilic zones were tested for the presence of aldehyde groups by adding 2 µL of 2,4-dinitrophenylhydrazine and observing the change in color from yellow to orange (Figure S1). The sheets of aldehyde-functionalized paper were stored in a desiccator until use.

Preparation of eosin-conjugated reporter antibody

The method of conjugation of EITC to proteins has been described previously.^[3,4] EITC (1 mg) was dissolved in 100 µL of DMSO. 20 µL of the above solution was mixed with a 400 µL solution (5.3 mg/mL) of the reporter antibody in 0.1 M sodium bicarbonate buffer (pH 9.0) to give a total reaction volume of 420 µL. The reaction mixture was protected from light and placed at 4 °C for five hours. During the reaction, the isothiocyanate functional group of EITC reacts with the amine group of the lysine residues of the antibody to form a thiourea bond (Figure S2, A). At the end of the reaction, the excess EITC was separated from the eosin-conjugated reporter antibody by size-exclusion columns with a Sephadex matrix (PD-10 Desalting Column and Micro G-25 Spin-Column).

UV–visible absorbance spectroscopy was used to determine the average number of eosin molecules coupled to each reporter antibody molecule by taking an absorbance spectrum of the eosin-conjugated reporter antibody (Figure S2, B) and using the following equation:

$$n_{EITC}/n_{Rep.Ab.} = (Abs_{525}/\epsilon_{EITC,525})/[\{Abs_{280} - (Abs_{525}\epsilon_{EITC,280}/\epsilon_{EITC,525})\}/\epsilon_{Rep.Ab,280}]$$

where n_{EITC} is the number of molecules of eosin, $n_{Rep.Ab}$ is the number of molecules of the reporter antibody, Abs_{280} and Abs_{525} are the measured absorbance values at 280 nm and 525 nm, respectively, $\epsilon_{EITC,525} = 90,200 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{EITC,280} = 26,800 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{Rep.Ab,280} = 280,200 \text{ M}^{-1}\text{cm}^{-1}$. After purification and characterization, the eosin-conjugated reporter antibody was diluted with glycerol to make a 50% v/v glycerol stock and stored in 10 µL aliquots at -20 °C until use.

Preparation of buffers

100 mL of 10X PBS was diluted with 900 mL of deionized H₂O to make 1X PBS solution. 1 g BSA was dissolved in 100 mL of 1X PBS to make a 1%w/v BSA solution (1% PBSA). 3.025 g Tris and 4.28 g NaCl were added to 450 mL diH₂O and the pH was adjusted to 7.5 by adding 2N HCl to give a 50 mM Tris-Cl solution (1X TBS).

Preparation of paper to capture PfHRP2

The hydrophilic test zones of the aldehyde-functionalized paper were used for the detection of PfHRP2 using a sandwich immunoassay. For ease-of-use, strips of paper containing four test zones each (2.8 cm × 1.5 cm) were cut from the sheets of the oxidized chromatography paper. We used a flow-through system^[5] where both the top and the bottom surfaces of the test zones were open to the atmosphere. Therefore, during the incubation steps, the paper strips were suspended in air to prevent wicking of the solutions from the test zone. This was accomplished by placing each end of a paper strip on the lid of a 0.5 ml centrifuge tube that was fitted inside the frame of an empty pipette-tip box. Each tube extended upwards from the frame and created a raised support for the paper strip. The test zones on each paper strip were thus completely suspended in air between two supports. Multiple centrifuge tubes were fitted into one pipette-tip box to enable simultaneous incubation of 10 strips of paper (40 test zones) in every box. The boxes were kept humid by partially filling them with diH₂O and keeping their lids closed during the incubation steps. The test zones to be prepared for the immunoassay were placed on supports, as described above, inside a humid pipette-tip box. A stock solution of the capture antibody was diluted to 67 μM (1 mg/ml) using 1X PBS and glycerol was added to a final concentration of 10%v/v. 2 μL of the above solution was added to each test zone and incubated overnight. After the incubation, the remaining solution of the capture antibody on the surface was wicked by bringing the bottom of the test zone in contact with a blotting paper. Each test zone was then washed with 40 μL of 1X PBS (two washes of 20 μL each (2 × 20μL)) by adding the wash solution to the top of the test zone and pressing the bottom surface against a blotting paper to wick the solution. To quench excess aldehyde groups that did not react with capture antibodies, each test zone was then incubated with 10 μL of 1X TBS in a humid pipette-tip box. The excess

solution was wicked into the blotting paper and the test zones were washed with 40 μL of 1X PBS ($2 \times 20 \mu\text{L}$).

Capture of PfHRP2

10 μL of a dilution of PfHRP2 (prepared in either 1% PBSA or human serum) was pipetted onto a test zone and incubated in a humid pipette-tip box for 30 minutes. As a negative control, a test zone was incubated with 10 μL of either 1% PBSA or undiluted human serum, without any PfHRP2, for the same duration. At the end of the incubation, the excess solution was wicked into blotting paper and each test zone was washed with 40 μL of 1X PBS ($2 \times 20 \mu\text{L}$).

Detecting the presence of PfHRP2

A 330 nM (50 $\mu\text{g/mL}$) solution of the eosin conjugated reporter antibody was prepared using 1% PBSA. Each test zone that was contacted with a sample (with or without PfHRP2) was incubated with 5 μL of the above solution in a humid pipette-tip box covered in foil for 30 minutes. At the end, the excess solution was wicked and each test zone was washed sequentially with PBST (1X PBS, 0.1%v/v Tween-20) ($1 \times 20 \mu\text{L}$), 1X PBS ($1 \times 20 \mu\text{L}$) and diH₂O ($1 \times 20 \mu\text{L}$). The presence of PfHRP2 was detected by measuring the fluorescence of eosin, followed by polymerization-based amplification.

Fluorescence imaging

Fluorescence microscopy was used to detect the presence of eosin on the surface of the paper during the development of the immunoassay. Each test zone was imaged using an Olympus LX81 microscope with a 4X objective lens, a 10X eyepiece lens, and a Semrock TxRed-4040C filter set using an exposure time of one second. A Lumen 200pro lamp with a Prior Lumen Bulb (Item #P-LM200BI) was used as the source of excitation light. The mean fluorescence intensity of each test zone was calculated by averaging the constituent pixel intensities using ImageJ (a public domain, Java-based image processing software).

Polymerization-based amplification (PBA)

An aqueous amplification solution containing 200 mM PEGDA, 150 mM TEA, 100 mM VP, 0.35 μ M eosin Y, 1.6 mM phenolphthalein and 20 mM hydrochloric acid (HCl) was prepared. Without the addition of HCl, the pH of the solution was 9.3 and phenolphthalein was present in its dianionic state (Figure S3) that strongly absorbs at the same wavelengths as eosin (Figure S4) and interferes with the initiation reaction (Supplementary Discussion, Section 1) Therefore, HCl was added to adjust the pH of the solution to 7.9. At this pH, phenolphthalein is colorless and does not interfere with the absorption of light by eosin (Figure S4). Each paper strip containing four test zones was cut to give four rectangular pieces (0.6 cm $\times$ 1.5 cm) containing one test zone in the middle of each piece. Each test zone was processed individually. The test zone was suspended in air by placing each end of its long side on a raised support built by attaching two smaller pieces of glass on top of a microscope slide. 20 μ L of the aqueous amplification solution was added to the test zone and the slide was placed on a specially-designed frame such that the test zone was positioned at a fixed distance ($\sim$ 9 cm) below an array of light-emitting diodes (LED) in an ampliPHOX® Reader (InDevR). The test zone containing the aqueous solution was then illuminated from above with a 522 nm light at 30 mW/cm² for a specified duration (see text). After irradiation was complete, the test zone was rinsed with diH₂O from a spray bottle, followed by a diH₂O wash (2 X 20 μ L) on the blotting paper.

Visualization and imaging of interfacial hydrogels on paper

2 μ L of 0.5 M NaOH was added to a test zone for visualization of the result. The results were imaged using a smartphone (HTC One™ mini). The images, except where specified, were taken immediately after the addition of NaOH. All images were used without any modification.

Quantification of colorimetric intensity and calculation of LoD

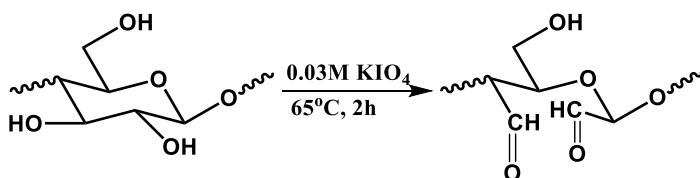
ImageJ was used to quantify the intensity of the colorimetric results on paper. A detailed procedure for quantification is given in Table S1.

The colorimetric intensity data (y), calculated for the dose-response trials of *Pf*HRP2 in a buffered solution, were fit to a sigmoidal curve using the function ‘nlinfit’ in Matlab. We

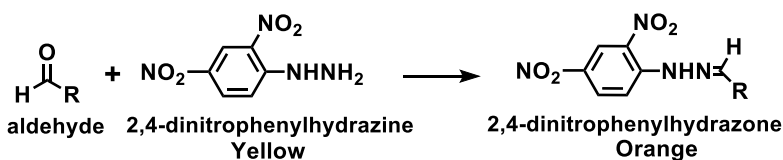
used a four-parameter equation: $y = (A - D)/(1 + (c/B)^n) + D$, where c is the concentration of PfHRP2, A is the lower asymptote, B , is the concentration at inflexion point, n is the slope at inflexion point, and D is the upper asymptote.^[6] The R-squared value for the fitted curve was 0.996 and the fitted parameters with 95% confidence intervals were calculated as follows: $A = 4.0$ (-0.9, 9.0), $B = 18.8$ (14.3, 23.3), $D = 96.0$ (85.4, 106.7), and $n = 1.4$ (1.0, 1.8).

Supplementary Figure S1: Preparation and characterization of aldehyde-functionalized paper (A) Selective oxidation of the C2-C3 vicinal hydroxyl groups in the glucose unit of cellulose by an aqueous solution of potassium periodate to give a dialdehyde product (aldehyde-functionalized paper). (B) Schematic of the reaction of 2,4-dinitrophenylhydrazine with an aldehyde moiety of the aldehyde-functionalized paper. (C) Results after unmodified and aldehyde-functionalized paper are reacted with 2,4-dinitrophenylhydrazine. Aldehyde-functionalized paper reacts to give a deep orange color and unmodified paper remains yellow.

(A) Functionalization of paper surface



(B) Colorimetric test for carbonyl compounds



(C) Confirmation of functionalization of paper using 2,4-dinitrophenylhydrazine



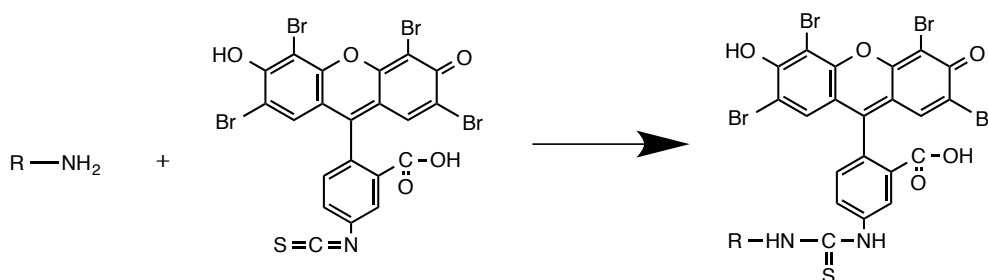
Unmodified paper
Yellow



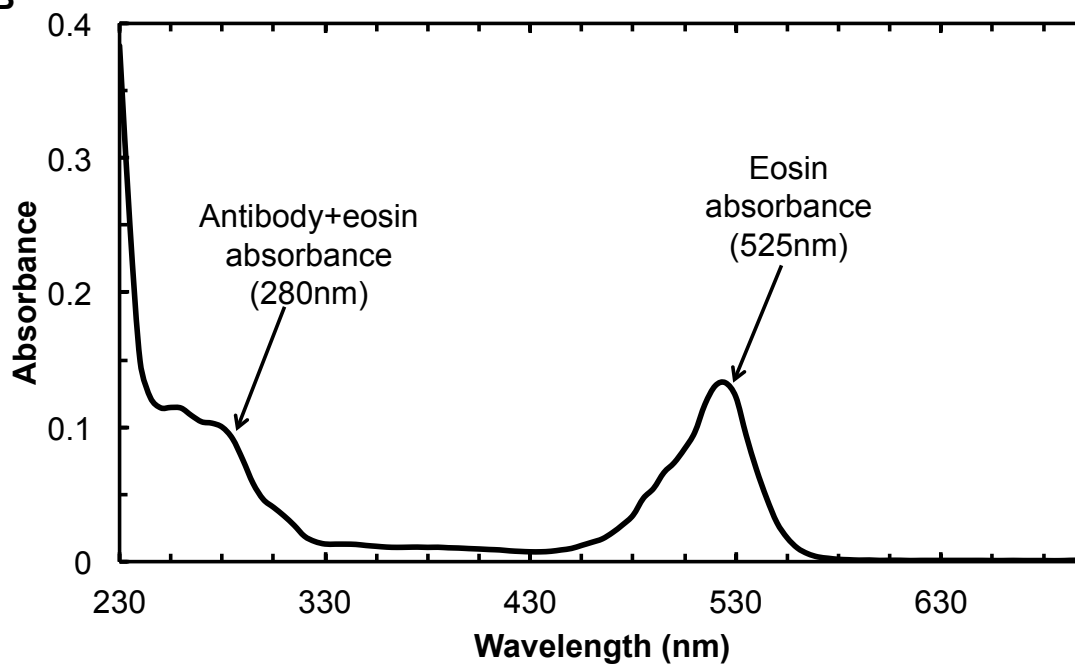
Aldehyde-functionalized paper
Orange

Supplementary Figure S2: Preparation and characterization of eosin-modified reporter antibodies. (A) Schematic of the reaction between the isothiocyanate group of eosin 5-isothiocyanate and an amine group of a lysine residue of the reporter antibody to form a thiourea bond. (B) A typical UV-visible absorption spectrum for purified, eosin-conjugated anti-*Pf*HRP2 reporter antibody

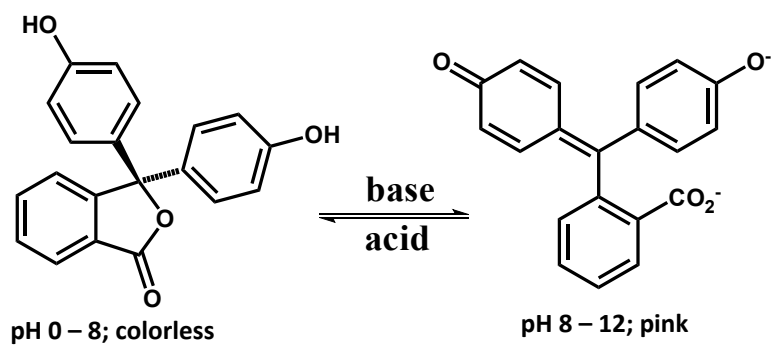
A



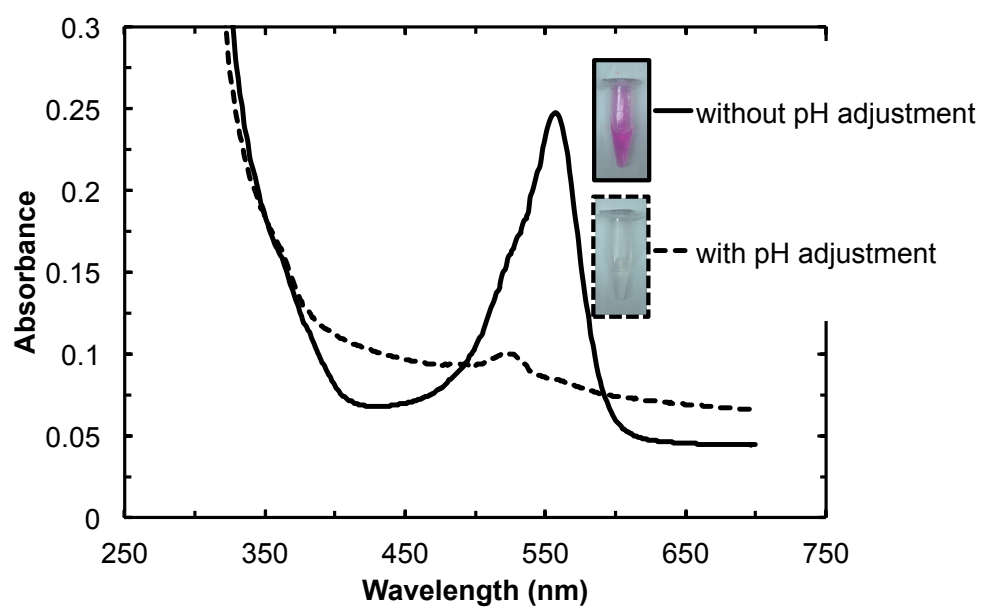
B



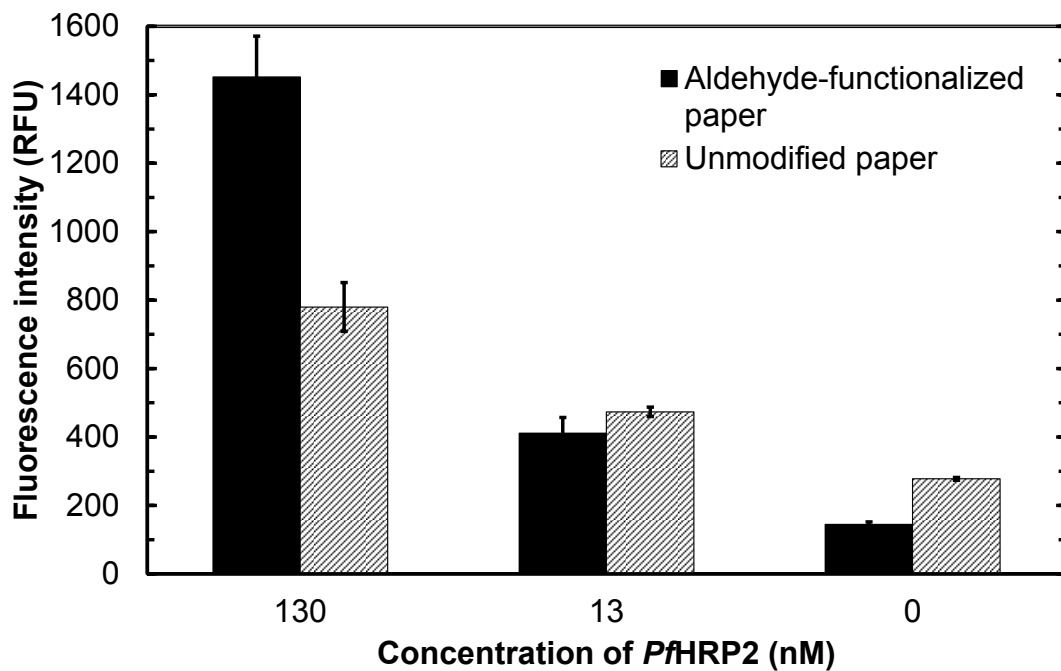
Supplementary Figure S3: Equilibrium between colorless and pink isomers of phenolphthalein as a function of pH



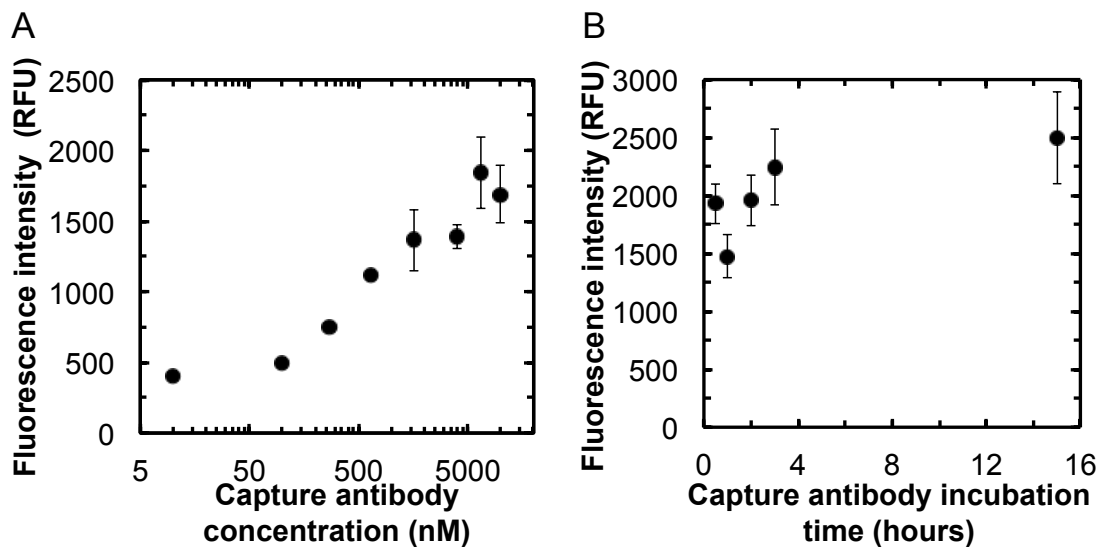
Supplementary Figure S4: UV-visible absorption spectra of aqueous amplification solution with (dashed line) and without (solid black line) pH adjustment.



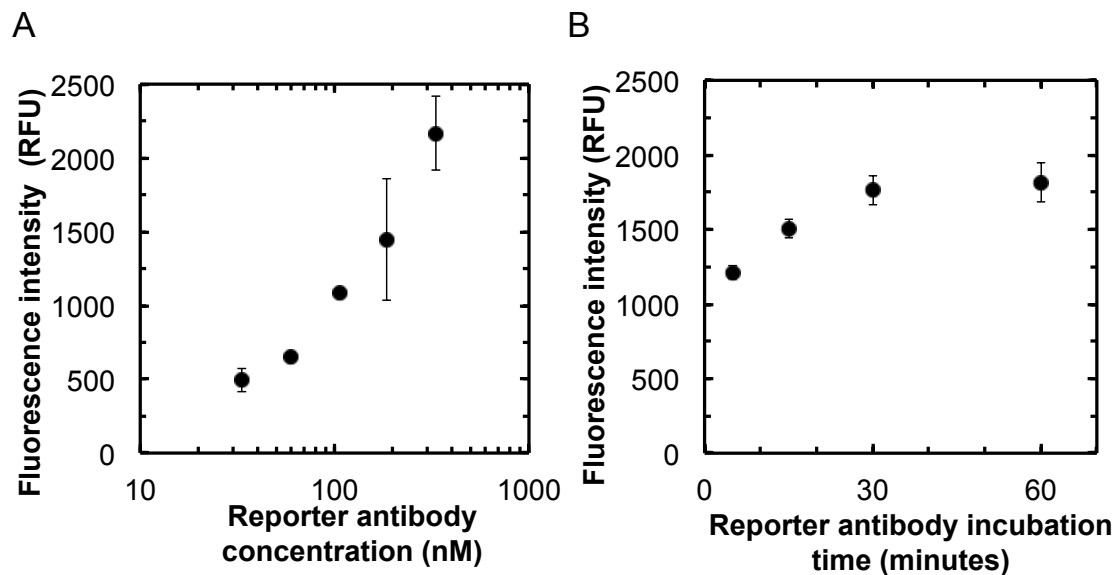
Supplementary Figure S5: Mean fluorescence intensity values for the detection of *Pf*HRP2 in a buffered solution using aldehyde-functionalized and unmodified Whatman No. 1 chromatography paper. The concentration (67 μ M) and the incubation time (overnight) of the capture antibody, the concentration (130 nM) and the incubation time (30 minutes) of *Pf*HRP2, and the concentration (330 nM) and the incubation time (30 minutes) of the eosin-conjugated reporter antibody were kept the same for both surfaces. The aldehyde-functionalized paper serves as a better surface for the detection of *Pf*HRP2 (with higher specific signal from surfaces that were contacted with *Pf*HRP2 and lower non-specific signal from negative controls) compared to the unmodified paper. Each bar is an average of four replicates and error bars denote standard deviation.



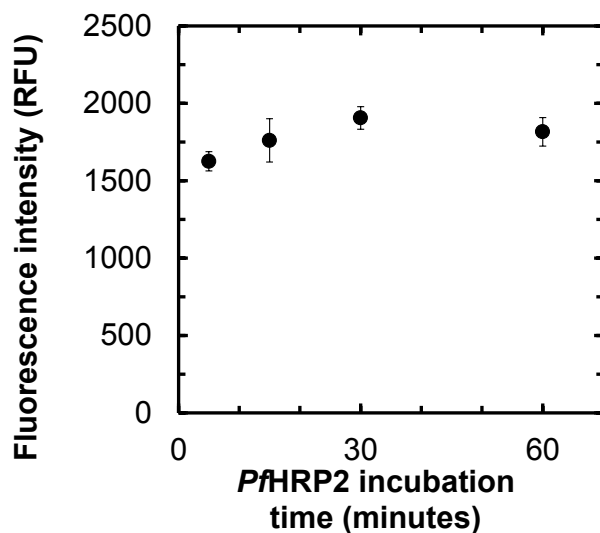
Supplementary Figure S6: Mean fluorescence intensity values for the detection of *Pf*HRP2 using paper where the concentration and the incubation time of the capture antibody were varied. (A) Different concentrations of capture antibody were incubated overnight (15 hours). (B) 67 μ M capture antibody was incubated for different times. The concentration (130 nM) and the incubation time (30 minutes) of *Pf*HRP2 and the concentration (330 nM) and the incubation time (30 minutes) of the eosin-conjugated reporter antibody were kept the same for both assays. Each data point is an average of three replicates and error bars denote standard deviation. The fluorescence intensity of surfaces that were contacted with samples without any *Pf*HRP2 was measured as a negative control (182 ± 18 RFU) for non-specific binding of the eosin-conjugated reporter antibody to the capture antibody. The fluorescence intensity of the surfaces that were not contacted with capture antibody, but were incubated with 130 nM *Pf*HRP2 and 330 nM eosin-conjugated reporter antibody was also measured (273 ± 72 RFU) as a negative control for non-specific binding of *Pf*HRP2 to the surface of the paper without any capture molecules. 67 μ M was chosen as the concentration of the capture antibody for the assays because it gave the highest fluorescent signal. For the incubation time of the capture antibody, the improvement in signal was minimal for an increase in time from 3 hours to 15 hours.



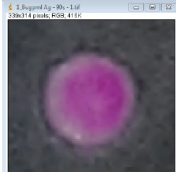
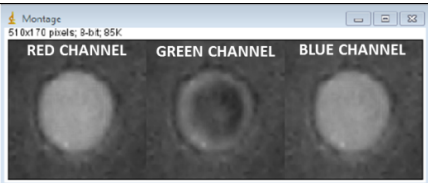
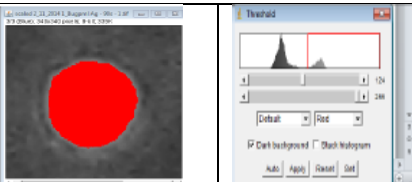
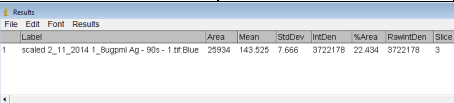
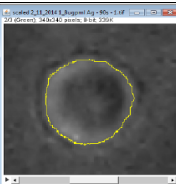
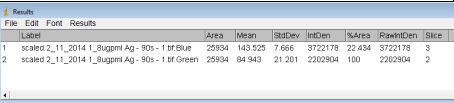
Supplementary Figure S7: Mean fluorescence intensity values for the detection of *Pf*HRP2 using paper where the concentration and the incubation time of the eosin-conjugated reporter antibody were varied. The concentration (67 μ M) and the incubation time (overnight) of the capture antibody and the concentration (130 nM) and the incubation time (30 minutes) of *Pf*HRP2 were kept the same for both assays. (A) Different concentrations of eosin-conjugated reporter antibody were incubated for 30 minutes. (B) 330 nM eosin-conjugated reporter antibody was incubated for different times. Each data point is an average of three replicates and error bars denote standard deviation. Fluorescence intensity of the surfaces that were prepared by an overnight incubation of 67 μ M capture antibody were reacted with 1% PBSA without any *Pf*HRP2 and contacted with the highest concentration of the eosin-conjugated reporter antibody (330 nM) for the longest incubation time (60 minutes) as a negative control (226 $\pm$ 16 RFU) for non-specific binding of the reporter antibody to the capture antibody. Increasing the concentration of the reporter antibody significantly increases the fluorescent signal. Therefore, the highest possible concentration of the reporter antibody (330 nM) was used in the assays. The effect of the increase in incubation time of the reporter antibody was observed up to 30 minutes after which an increase in the incubation time led to a minimal increase in the fluorescent intensity.



Supplementary Figure S8: Mean fluorescence intensity values for the detection of *Pf*HRP2 on paper in which sample (containing *Pf*HRP2) incubation time was varied. 67 μ M capture antibody was incubated overnight (15 hours). Next, a sample containing 130 nM *Pf*HRP2 was incubated for different times, following which 330 nM eosin-conjugated reporter antibody was incubated for 30 minutes. Each data point is an average of three replicates and error bars denote standard deviation. The effect of the incubation time of *Pf*HRP2 was seen up to 10 minutes after which an increase in the incubation time led to a minimal increase in the fluorescence intensity.



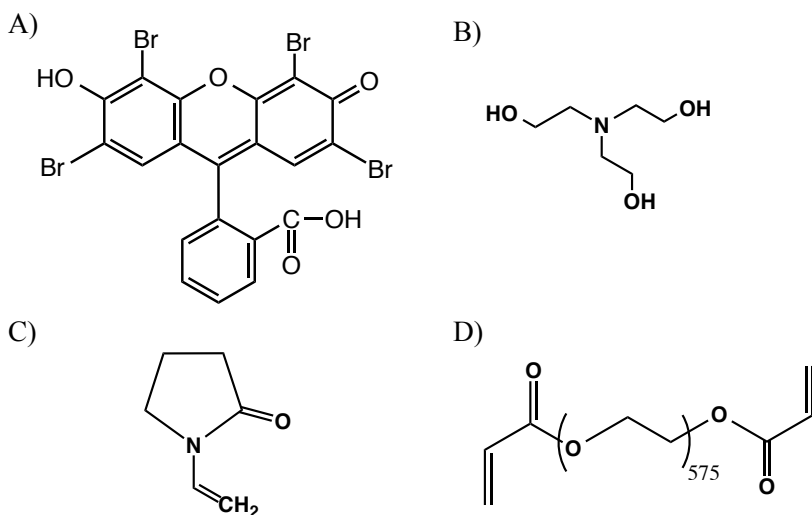
Supplementary Table S1: Procedure for quantification of colorimetric result using ImageJ.

Task	Command	Results																								
1. Open the image in ImageJ																										
2. Convert the image into red, green and blue channels	Image -> Type -> RGB stack																									
3. Select the blue channel and threshold it. Select upper and lower limits to ensure that only the test zone is selected. Do not select “apply”	(Click on blue channel to select it) Image -> Adjust -> Threshold																									
4. Measure the average intensity of the area that is thresholded	Analyze -> Measure	 <table><thead><tr><th>Label</th><th>Area</th><th>Mean</th><th>StdDev</th><th>IntDen</th><th>%Area</th><th>RawIntDen</th><th>Slice</th></tr></thead><tbody><tr><td>1 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifBlue</td><td>25934</td><td>143.525</td><td>7.666</td><td>3722178</td><td>22.434</td><td>3722178</td><td>3</td></tr></tbody></table>	Label	Area	Mean	StdDev	IntDen	%Area	RawIntDen	Slice	1 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifBlue	25934	143.525	7.666	3722178	22.434	3722178	3								
Label	Area	Mean	StdDev	IntDen	%Area	RawIntDen	Slice																			
1 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifBlue	25934	143.525	7.666	3722178	22.434	3722178	3																			
5. (a) Select the area that is thresholded in the blue channel to define the boundary of the test zone (b) Remove the threshold from the image and select the green channel	(a) Edit -> Selection -> Create selection (b) Image -> Adjust -> Threshold -> Reset (Click on the green channel to select it)																									
6. Measure the average intensity of the test zone in the green channel	Analyze -> Measure	 <table><thead><tr><th>Label</th><th>Area</th><th>Mean</th><th>StdDev</th><th>IntDen</th><th>%Area</th><th>RawIntDen</th><th>Slice</th></tr></thead><tbody><tr><td>1 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifBlue</td><td>25934</td><td>143.525</td><td>7.666</td><td>3722178</td><td>22.434</td><td>3722178</td><td>3</td></tr><tr><td>2 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifGreen</td><td>25934</td><td>84.943</td><td>21.201</td><td>2202904</td><td>100</td><td>2202904</td><td>2</td></tr></tbody></table>	Label	Area	Mean	StdDev	IntDen	%Area	RawIntDen	Slice	1 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifBlue	25934	143.525	7.666	3722178	22.434	3722178	3	2 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifGreen	25934	84.943	21.201	2202904	100	2202904	2
Label	Area	Mean	StdDev	IntDen	%Area	RawIntDen	Slice																			
1 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifBlue	25934	143.525	7.666	3722178	22.434	3722178	3																			
2 scaled_2_11_2014_1_Sugmi Ag - 90s - 1.tifGreen	25934	84.943	21.201	2202904	100	2202904	2																			
7. Subtract the average intensity in the green channel from the average intensity in the blue channel																										

Supplementary discussion

1. Eosin-mediated polymerization reaction and visualization of interfacial hydrogels

This section summarizes the current understanding of the mechanism for co-polymerization of the acrylate monomers, PEGDA and VP, using an eosin/tertiary amine photoinitiation system. The structures of the important reaction components are shown in Figure DS1.

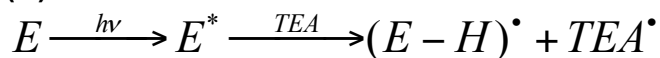


Supplementary Discussion Figure DS1: (A) eosin Y, (B) triethanolamine (TEA), (C) 1-vinyl-2-pyrrolidinone (VP), and (D) poly(ethylene glycol) diacrylate (PEGDA)

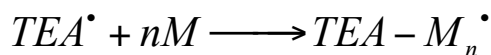
The use of xanthene dyes in photoinitiation systems has been studied extensively.^[7-9] The eosin/tertiary amine photoinitiation system in particular has historically been used for the polymerization of acrylate monomers for encapsulation of cells.^[10] When irradiated with green light, the triplet excited state of eosin (E^*) accepts an electron from TEA. The TEA radical cation then loses a proton to generate the initiating TEA radical.^[9,11,12] The initiating radical can then react with an acrylate monomer (PEGDA or VP) to generate a propagating polymer chain. The termination of the polymer chains can occur either through combination or disproportionation reactions between the radical ends of the growing polymer chains. If dissolved oxygen is present, it reacts with initiating radicals and the radical termini of growing polymer chains at a diffusion-limited rate to generate less reactive peroxide radicals. The peroxide radicals are unreactive towards further

propagation and thus terminate the growth of the polymer chains.^[13] Therefore, free radical polymerization reactions in PBA were previously carried out under oxygen-free conditions by purging the system with an inert gas.^[14] However, Avens et al. recently proposed a mechanism by which a series of disproportionation termination reactions between the semi-reduced eosin (E-H) radicals and peroxide radicals can cyclically regenerate eosin and the polymerization reaction can proceed in the presence of oxygen.^[15] This observation for solution phase, bulk polymerizations was used to develop an interfacial PBA system (i.e. polymerization only in areas where binding events have occurred) that does not require purging the system with an inert gas.^[4] We are currently investigating the mechanism of this eosin-based polymerization reaction in the presence of oxygen using spectroscopy in combination with kinetic modeling. The reaction scheme presented in Figure DS2 consists of reactions that are thought to be the most important on the basis of the existing literature.

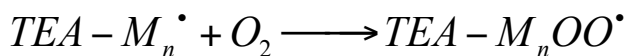
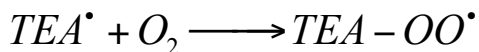
(A) Initiation



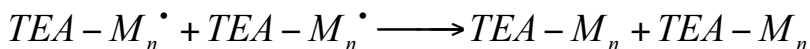
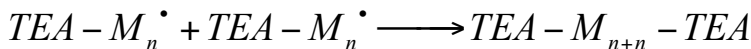
(B) Propagation



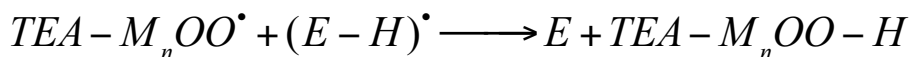
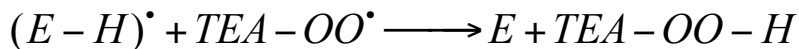
(C) Inhibition



(D) Termination



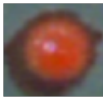
(E) Eosin regeneration and termination



Supplementary Discussion Figure DS2: Eosin-mediated polymerization: initiation, propagation, inhibition, termination and eosin re-generation reactions

The hydrogel formed by polymerization of acrylate monomers is transparent; therefore, a visualization strategy is needed to detect the presence of the interfacial hydrogel in assays using PBA. On bioactive glass surfaces, the hydrogels are typically swollen with a dye solution. This technique results in high-contrast between the hydrogel and the bare glass because the dye swells into the hydrogel but does not stain the surface of the glass. With paper, however, the dye solution adheres to the surface non-specifically even after thorough washing. Therefore, it is difficult to differentiate the hydrogel from the background of the paper, which would increase the likelihood of false positive interpretations in a diagnostic device.

A) Paper surface with hydrogel



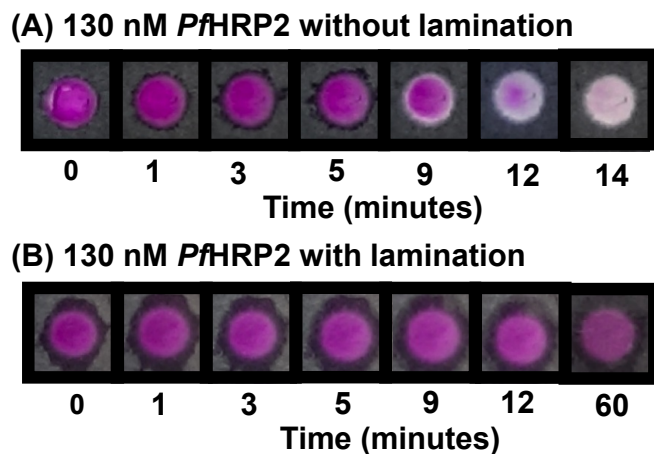
B) Paper surface without hydrogel



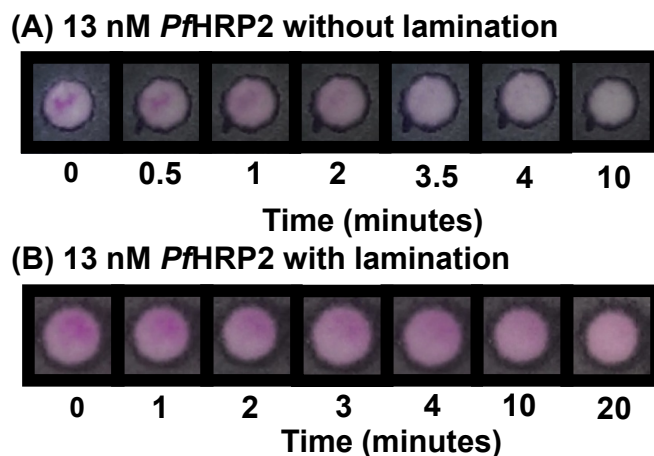
Supplementary Discussion Figure DS3: Visualization on paper (A) with hydrogel, and (B) without hydrogel, by swelling with a dye solution. The dye solution adheres non-specifically to paper, thus the colorimetric result is similar regardless of the presence of the hydrogel.

2. Stability of color on paper after addition of NaOH for visualization

The change in color of phenolphthalein that gets trapped in the cross-linked hydrogel formed at the surface of a paper test zone occurs immediately upon the addition of NaOH to the surface ($t=0$, Figures DS4 and DS5) and allows a user to interpret the result of the test without any waiting time. As time increases, the color becomes progressively faint and eventually disappears. The fading is primarily due to two reasons, evaporation of the NaOH solution (phenolphthalein is colorless in dry state), and diffusion of phenolphthalein out of the hydrogel. The time required for the color to fade depends on the initial intensity of the colorimetric response that is dependent on the amount of phenolphthalein trapped in the hydrogel, which in turn depends on the thickness of the hydrogel. For thick hydrogels formed on the surfaces contacted with a high concentration of *PfHRP2* (130 nM), the pink color persisted for at least 10 minutes until the NaOH solution evaporated (Figure DS4, A). We prevented evaporation after addition of NaOH to the test zone by tightly wrapping a piece of scotch tape on both sides of the paper to completely seal it (lamination). Under this condition, the color persisted for more than 60 minutes (Figure DS4, B). For thin hydrogel films formed from a lower concentration of *PfHRP2* (13 nM), the diffusion of phenolphthalein from the hydrogel occurred before the evaporation of the NaOH solution (Figure DS5, A) and the color faded within 3 minutes of adding NaOH. For this case, lamination of the paper extended the stability of the color to more than 20 minutes (Figure DS5, B) by limiting the spread of the NaOH solution.



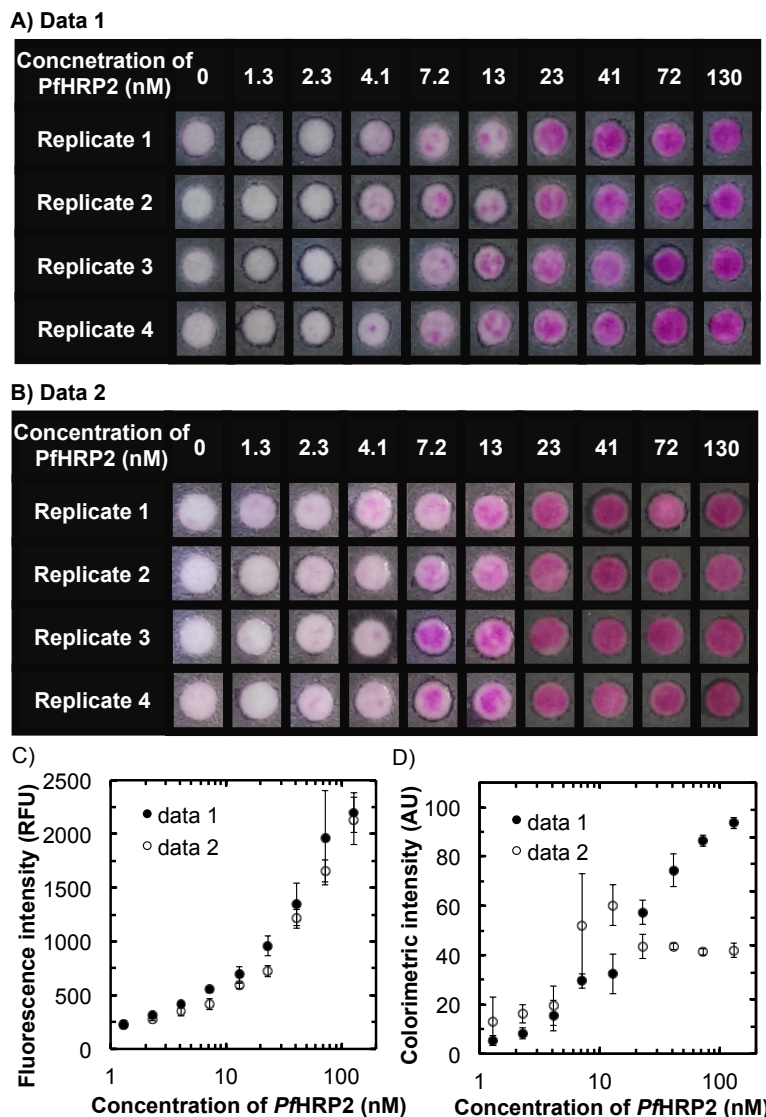
Supplementary Discussion Figure DS4: Images showing the colorimetric response for detection of 130 nM *Pf*HRP2 on paper (A) with, and (B) without lamination after addition of 2 μ L NaOH at t=0 minutes.



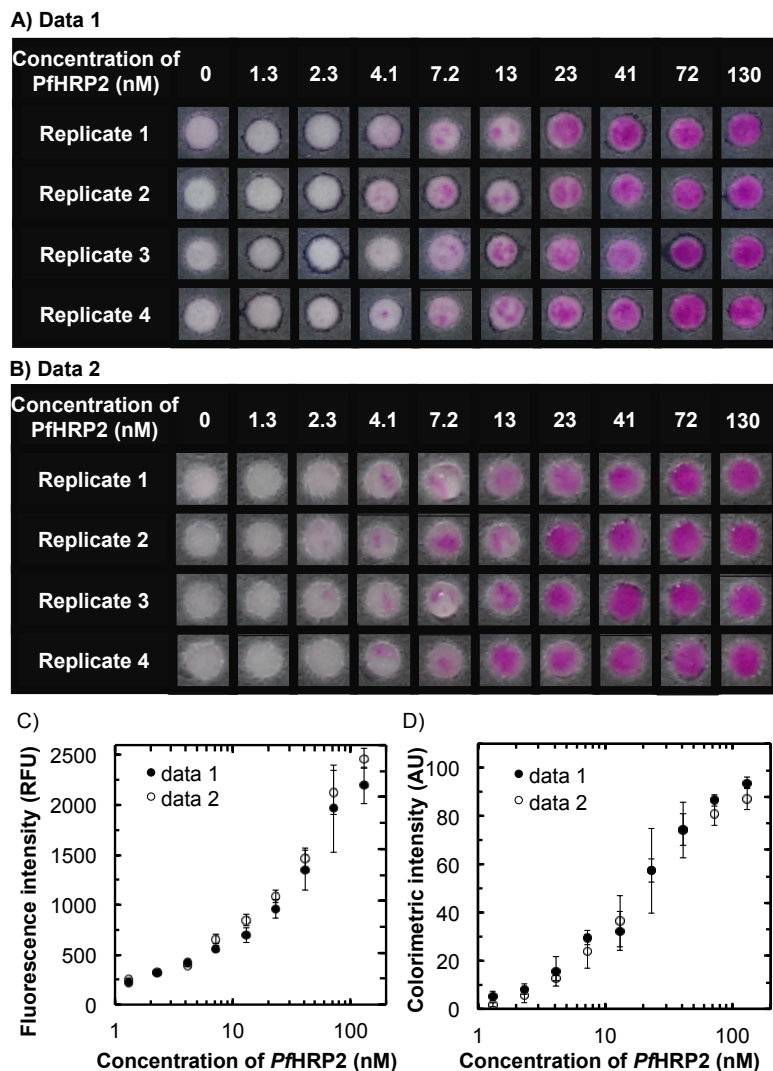
Supplementary Discussion Figure DS5: Images showing the colorimetric response for detection of 13 nM *Pf*HRP2 on paper (A) with, and (B) without lamination after addition of 2 μ L NaOH at t=0 minutes.

3. Effect of ambient light during imaging

Paper surfaces that were prepared by overnight incubation of 67 μ M capture antibody were contacted with different concentrations of *Pf*HRP2, ranging from 1.3 nM to 130 nM, in 1% PBSA. Negative test surfaces were contacted only with 1% PBSA. All of the above surfaces were contacted with 330 nM eosin-conjugated reporter antibody and imaged for fluorescence. The surfaces were then contacted with the aqueous amplification solution and irradiated with light for 90 seconds. They were washed to remove the unreacted monomer and visualized with 2 μ L of 0.5 M NaOH. The surfaces were imaged with or without the use of a desk lamp immediately after addition of NaOH and the color intensity was quantified using ImageJ. The procedure outlined above was repeated independently on three different days and the results were compared. Even though the fluorescence intensity data gave good agreement, indicating similar photoinitiator density on the surfaces that were contacted with same concentrations of *Pf*HRP2 in independent trials, non-uniformities in light during imaging (due to differences in ambient light at different times of the day) led to dramatically different color intensity values (Figure DS6). If the light falling on the surfaces during imaging was controlled by the use of an ordinary desk lamp, the color intensity data showed good agreement between independent trials (Figure DS7).



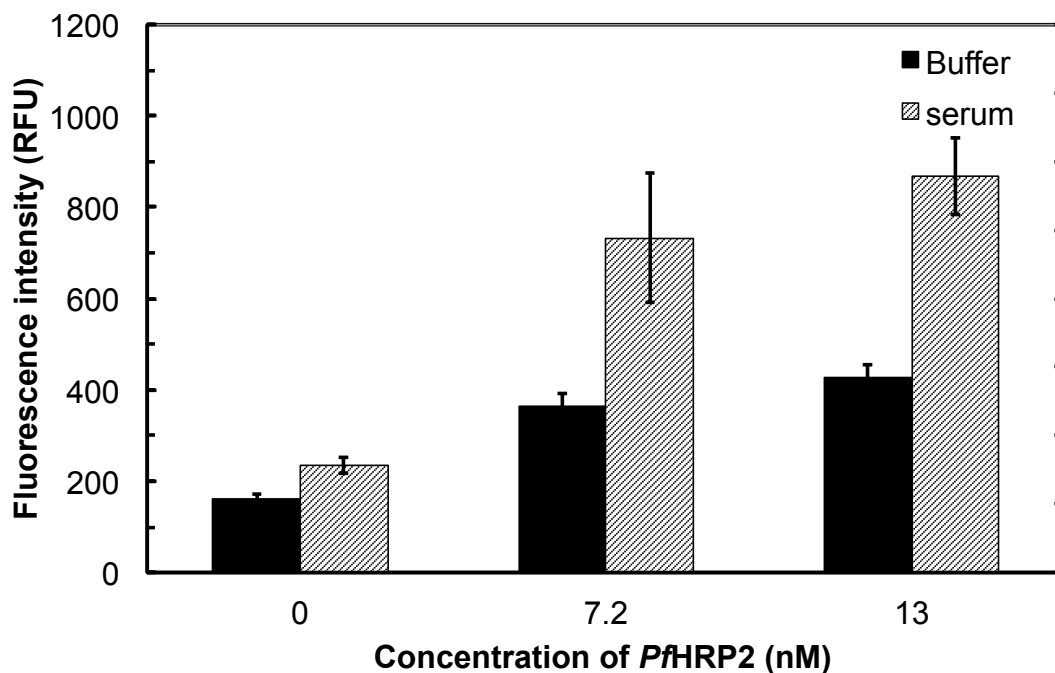
Supplementary Discussion Figure DS6: Images for a dose-response trial done on two different days, (A) and (B). The images in (A) were taken by illuminating the surfaces with an ordinary desk lamp and images in (B) were taken under ambient light. For (B), the surfaces with *Pf*HRP2 concentration of 0-13 nM were imaged during the day and surfaces with *Pf*HRP2 concentration of 23-130 nM were imaged after dark. (C) A comparison of fluorescence measurements taken before polymerization showed good agreement in the amount of surface-bound eosin for same concentrations of *Pf*HRP2 tested on different days. (D) Quantification of the colorimetric intensity using the images shown in (A) and (B) showed a dramatic difference in values for surfaces that were imaged after sunset.



Supplementary Discussion Figure DS7: Images for a dose-response trial done on two different days (A) and (B). The images for both trials were taken by illuminating the surfaces with an ordinary desk lamp to control the effect of differences in ambient light. (C) A comparison of fluorescence measurements taken before polymerization showed good agreement in the amount of surface-bound eosin for same concentrations of *PfHRP2* tested on different days. (D) Quantification of the colorimetric intensity using the images shown in (A) and (B) showed good agreement between results on different days when the effect of non-uniformities in ambient light was controlled.

4. Effect of a complex sample matrix on the performance of PBA on paper and reproducibility of the illumination time

Detection of *Pf*HRP2 in human serum rather than in a buffer solution did not increase the visual LoD (Figure 3). However, in order to differentiate between the positive samples and the negative controls, the illumination time for the polymerization reaction had to be reduced from 90 seconds for surfaces contacted with samples prepared in buffer to 50 seconds for surfaces contacted with samples prepared in serum. To account for this observed decrease, both positive and negative surfaces contacted with samples prepared separately in serum and buffer were compared using fluorescence measurements. We observed that the fluorescence intensity of the surfaces contacted with human serum was higher than the fluorescence intensity of the surfaces contacted with a buffer solution (Figure DS8). We attribute the difference to non-specific binding of proteins in the serum to the capture antibody and/or the surface of the paper followed by nonspecific binding of the eosin-labeled reporter antibody to these proteins. The presence of a higher eosin density on the negative serum samples required a decrease in the illumination time from 90 seconds to 50 seconds to prevent bulk polymerization on these surfaces.^[4]



Supplementary Discussion Figure DS8: Mean fluorescence intensities of surfaces tested with dilutions of *Pf*HRP2 prepared in buffer and in human serum.

For surfaces that were used to detect *Pf*HRP2 in a buffer solution, an illumination time of 90 seconds was reproducibly used to differentiate between negative controls and positive samples. We did five independent dose-response trials for *Pf*HRP2 detection in buffer samples and tested the effect of, i) two different batches of paper that were oxidized independently, and ii) two different lots of capture antibody purchased from the same manufacturer. In addition, the amplification solution, the dilutions of *Pf*HRP2 and the dilution of the eosin-conjugated reporter antibody were freshly prepared for each trial. We found that the required illumination time remained consistent across all trials. Similarly, a reaction time of 50 seconds was reproducibly used to detect *Pf*HRP2 in serum samples.

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