Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

Polymerization-based Signal Amplification for Paper-based Immunoassays

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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), respectively. 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 KIO_4 solution at 65 °C for 2 hours (Figure S1, A).[1] After the reaction, the sheets were washed three times by dipping them in fresh deionized water (diH_2O) for one minute each and pouring off the water at the end. After the last wash had been poured out, 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(Figure S1, B). The presence of the aldehyde groups in the test zones allowed us to covalently immobilize amine-containing molecules (such as the anti-*Pf*HRP2 capture antibody in this study) to the surface of the paper through a Schiff-base linkage (Figure S1, C). The presence of the aldehyde groups in the test zones was confirmed by adding 2 µL of 2,4-dinitrophenylhydrazine and observing the change in color from yellow to orange (Figure S2). 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 S3, 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 scan of the eosin-conjugated reporter antibody (Figure S3, B) and using the following equation:

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

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, $\varepsilon_{EITC,525} = 90{,}200\ M^{-1}cm^{-1}$, $\varepsilon_{EITC,280} = 26{,}800\ M^{-1}cm^{-1}$ and $\varepsilon_{Rep.Ab,280} = 280{,}200\ M^{-1}cm^{-1}$. The purified and characterized eosin-conjugated reporter antibody, containing an average of 7 eosin molecules per reporter antibody molecules, was diluted to make 50% v/v glycerol stock and stored in 10 µL aliquots at -20 °C until use.

Preparation of buffers and stock solutions

100 mL of 10X PBS was diluted with 900 mL of diH_2O 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 diH_2O and the pH was adjusted to 7.5 by adding 2N HCl to give a 50 mM Tris-Cl solution (1X TBS). 1 mg lyophilized *Pf*HRP2 was dissolved in 200 µL of 2% PBSA to get 64 µM (5 mg mL^{-1}) stock solution. 1.5 µL aliquots of the above solution were stored at -80 °C. The capture antibody, as received from the manufacturer, was stored in 10 µL aliquots at -20°C.

Capture of PfHRP2 on paper

The hydrophilic test zones of the aldehyde-functionalized paper were used for the detection of *Pf*HRP2 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 had to be suspended in air to prevent wicking of the solutions from the test zone. This layout 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 diH_2O 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 block the excess aldehyde groups against non-specific binding of proteins, each test zone was then incubated with 10 μL of 1X TBS in a humid pipette-tip box. The excess solution was wicked on the blotting paper and the test zones were washed with 40 μL of 1X PBS (2 × 20 μL). Following the wash, 10 μL solution of *Pf*HRP2 (prepared in either 1% PBSA or human serum) was pipetted on a test zone and incubated in a humid pipette-tip box for 30 minutes. For experiments in buffer, *Pf*HRP2 solutions were prepared by adding 1 μL stock solution of *Pf*HRP2 to 499 μL of 1% PBSA to make a 130 nM solution - the highest concentration used in the experiments. The 130 nM solution was diluted further with 1% PBSA to get the lower concentrations. For experiments in human serum, the solutions were prepared as above using undiluted serum instead of PBSA. For negative control, a test zone was incubated with 10 μL of either 1% PBSA or undiluted human serum, without any *Pf*HRP2, for the same duration. At the end of the incubation, the excess solution was wicked on a blotting paper and each test zone was washed with 40 μL of 1X PBS (2 × 20 μL).

Detecting the presence of PfHRP2

A 330 nM (50 μg/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 *Pf*HRP2) 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 × 20 μL), 1X PBS (1 × 20 μL) and diH_2O (1 × 20 μL). The presence of *Pf*HRP2 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 IX81 microscope with a 4X objective lens, a 10X eyepiece lens, and a Semrock TxRed-4040C filter set using an exposure time of one second. Lumen 200 with a Prior Lumen Bulb (Item #P-LM200BI) was used as the source of 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) on paper

An aqueous amplification solution containing 200 mM PEGDA, 150 mM TEA, 100 mM VP, 0.35 μM eosin Y, 1.6 mM phenolphthalein and 20 mM hydrochloric acid (HCl) was prepared. During polymerization, 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[4,6] and phenolphthalein and HCl are included for visualization. Phenolphthalein, a weak acid, is colorless at a pH less than 8. As the pH increases above 8, the equilibrium shifts in favor of the pink dianionic form (Figure S4). 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 amplification solution without pH adjustment, it is predominantly present in its dianionic state that absorbs strongly at the excitation wavelength of eosin, and thus competes with eosin for absorption of light during the initiation step. Therefore, HCl was added to adjust the pH of the solution to 7.9. At this pH, phenolphthalein is present in its acidic, colorless form and does not interfere with the absorption of light by eosin. We confirmed the state of phenolphthalein before and after adjustment of the pH using UV-visible spectroscopy (Figure S5). The concentration of phenolphthalein added to the solution was maximized within the limits of solubility to provide an intense colorimetric response.

Each paper strip containing four test zones was cut to give four rectangular pieces (0.6 cm × 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 μL 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 (~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/cm^2 for a specified duration. After irradiation was complete, the test zone was rinsed with diH_2O from a spray bottle, followed by a diH_2O wash (2 X 20 μL) on the blotting paper.

Visualization and imaging of interfacial hydrogels on paper

2 μL of 0.5 M NaOH was added to a test zone for visualization of the result. The results were imaged with a smartphone, HTC One™ mini, using its camera in the 'HDR' mode with default settings. The phone was mounted on a ring stand to stabilize it. An ordinary desk lamp was used to shine light on the surface to keep the lighting conditions consistent at different times of the day (See Supplementary discussion, 'Effect of ambient light during imaging'). A white sheet of A4 paper, with a slit cut through it for imaging, was also placed between the phone and the test surface to prevent glare from the overhead light and the lamp. The images were taken immediately after the addition of NaOH and transferred to a computer. These images were used without any modification.

Optimization of the immunoassay

We optimized the paper-based immunoassay using *Pf*HRP2 solutions prepared in 1% PBSA. The effect of variables such 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 (Figures S6-9).

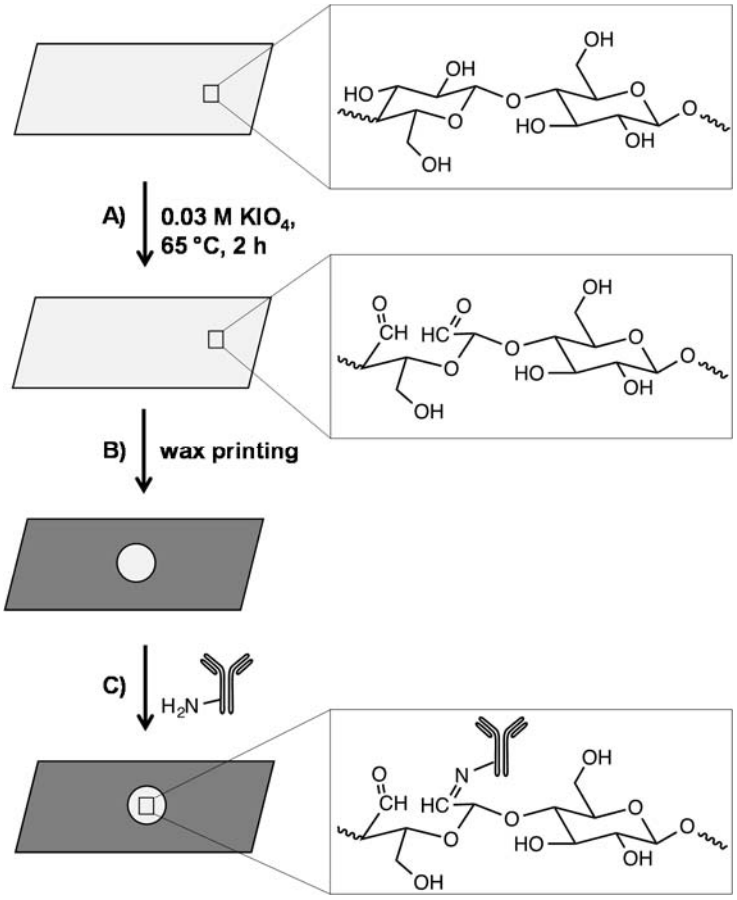
Quantification of colorimetric intensity and calculation of LoD

ImageJ was used to quantify the intensity of the colorimetric results on paper. A detailed procedure for quantification is given in Table S1. The calculated colorimetric intensity values for each concentration of *Pf*HRP2 and the p-values for a 1- tailed unpaired t-test between adjacent data points is given in Table S2. The analysis shows that all the concentrations of *Pf*HRP2 that were tested were statistically distinguishable from the control surfaces and from each other. 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 *Pf*HRP2, A is the lower asymptote, B, is the concentration in nM at inflexion point, n is the slope at inflexion point, and D is the upper asymptote.[7] 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).

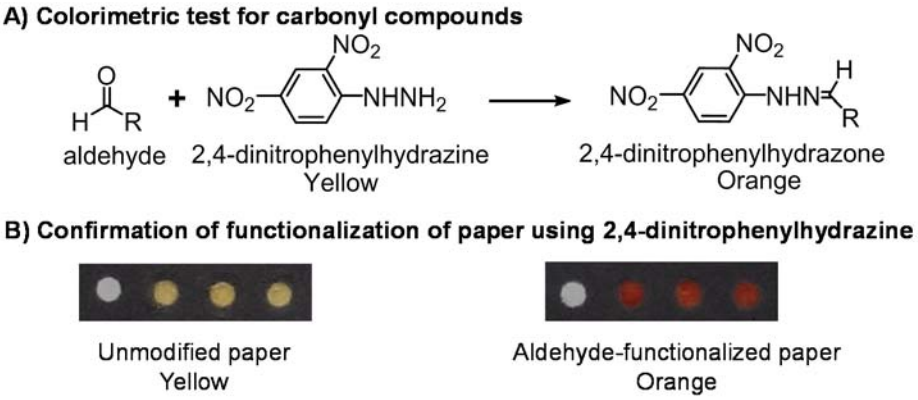
Storage experiments

The storage experiments were performed by preparing surfaces as detailed in the manuscript. During these experiments, the surfaces were stored in a closed drawer We believe that for storage before polymerization, it is important to protect the surfaces from light by storing them in a closed drawer, an envelope, an opaque box or any other container because we have the photoinitiator on the surface. The initiator tolerates ambient light during the wash steps in the assay without any special care to shield it, but for storage on the days and month timescale, it becomes important to shield it from light.[8] For storage after polymerization, even though the surfaces were stored in a closed drawer, we do not think that it is important to do so because for these surfaces only the phenolphthalein trapped in the hydrogel is important, and phenolphthalein is stable under ambient light.

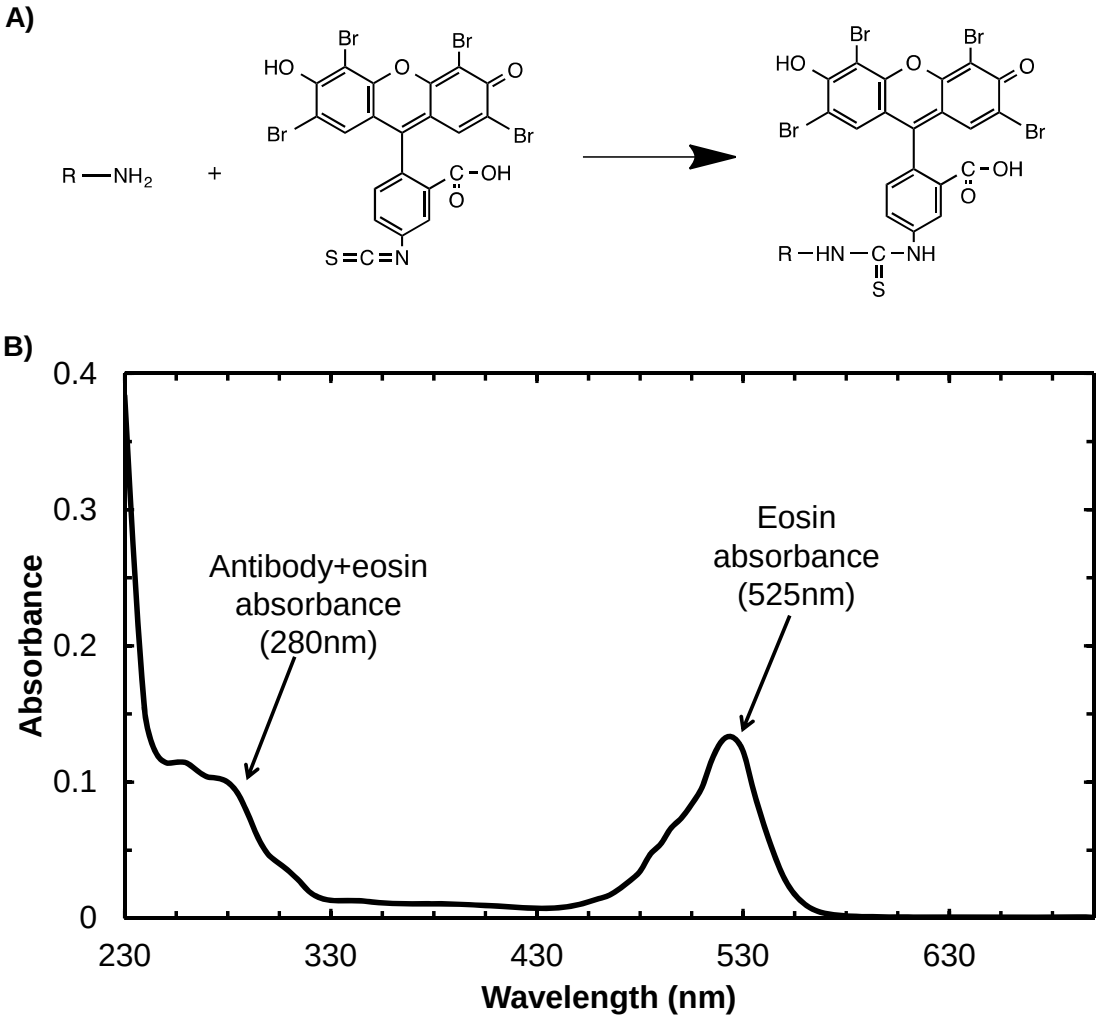
Supplementary Figure S1: Preparation of aldehyde-functionalized hydrophilic test zones on paper for covalent immobilization of amine-containing molecules (A) An aqueous solution of potassium periodate was used to selectively oxidize the C2-C3 vicinal hydroxyl groups in the glucose unit of cellulose to give a dialdehyde product (aldehyde-functionalized paper). (B) The modified paper was printed with wax and heated to create hydrophilic test zones surrounded by hydrophobic wax barriers. (C) Amine groups on a molecule (e.g. amine groups of the lysine residues of an antibody) can form a Schiff base linkage with the aldehyde groups to covalently immobilize the molecules on the surface of the paper.



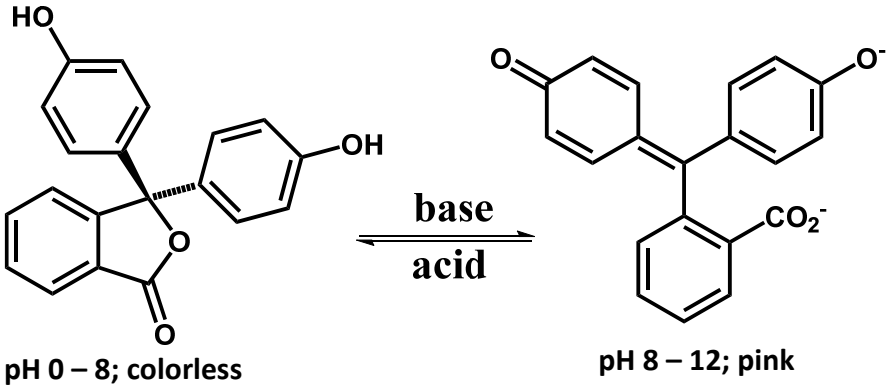
Supplementary Figure S2: Characterization of aldehyde-functionalized paper using 2,4 dinitrophenylhydrazine (2,4-DNP). (A) Schematic of the reaction of 2,4-DNP with an aldehyde moiety of the aldehyde-functionalized paper. (B) Results after unmodified and aldehyde-functionalized paper are reacted with 2,4-DNP. Aldehyde-functionalized paper reacts to give a deep orange color and unmodified paper remains yellow.



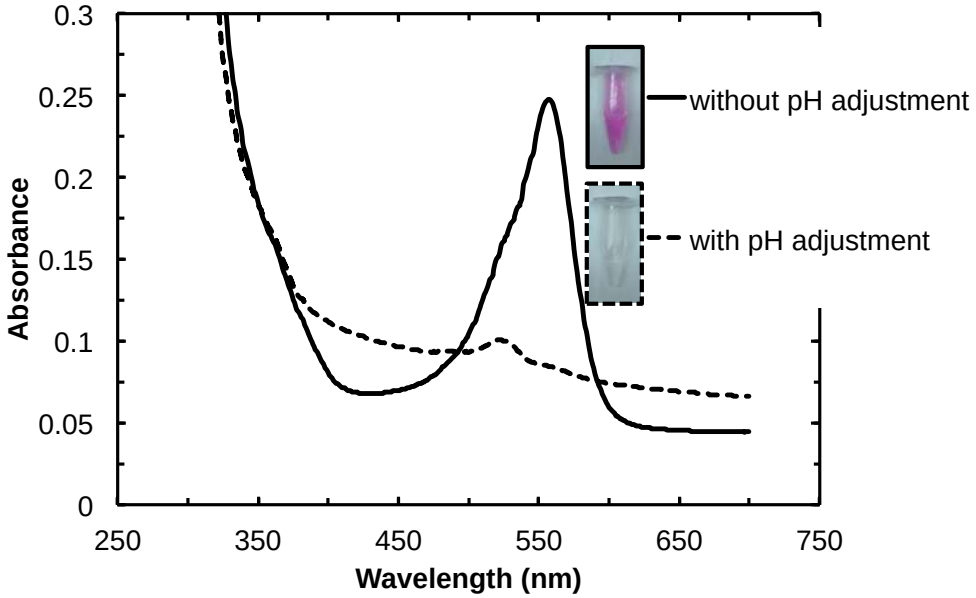
Supplementary Figure S3: Preparation and characterization of eosin-modified reporter antibody (A) Schematic of the reaction between an isothiocyanate group on eosin 5-isothiocyanate and an amine group of a lysine residue of the reporter antibody to form a thiourea bond. (B) A typical UV-visible absorption spectrum for purified, eosin-conjugated anti-*Pf*HRP2 reporter antibody.



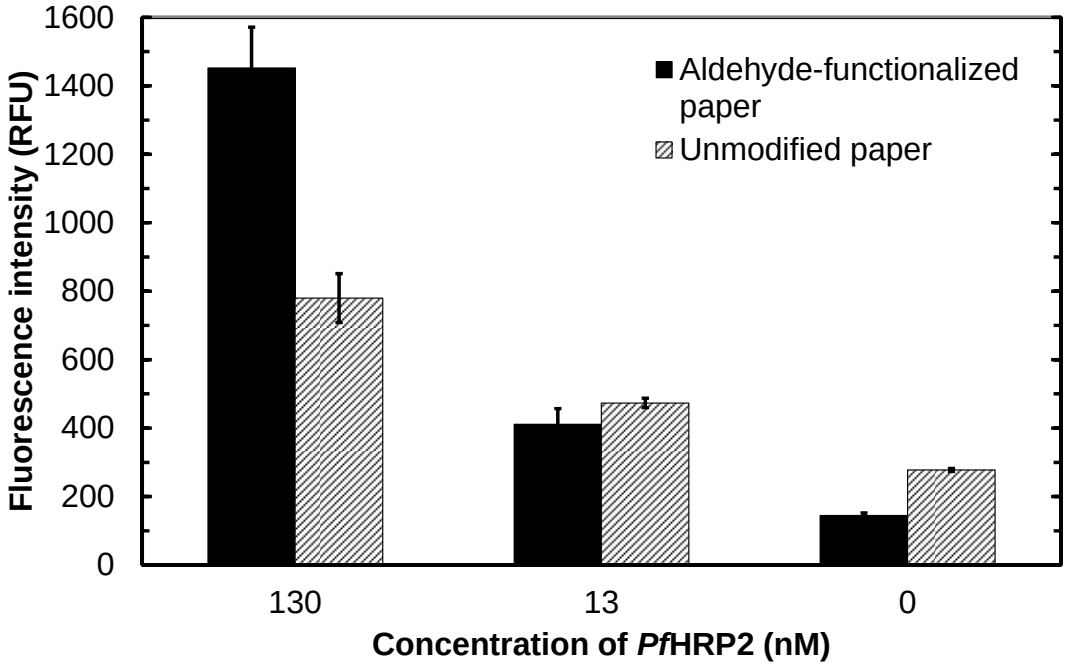
Supplementary Figure S4: Equilibrium between colorless and pink isomers of phenolphthalein as a function of pH.



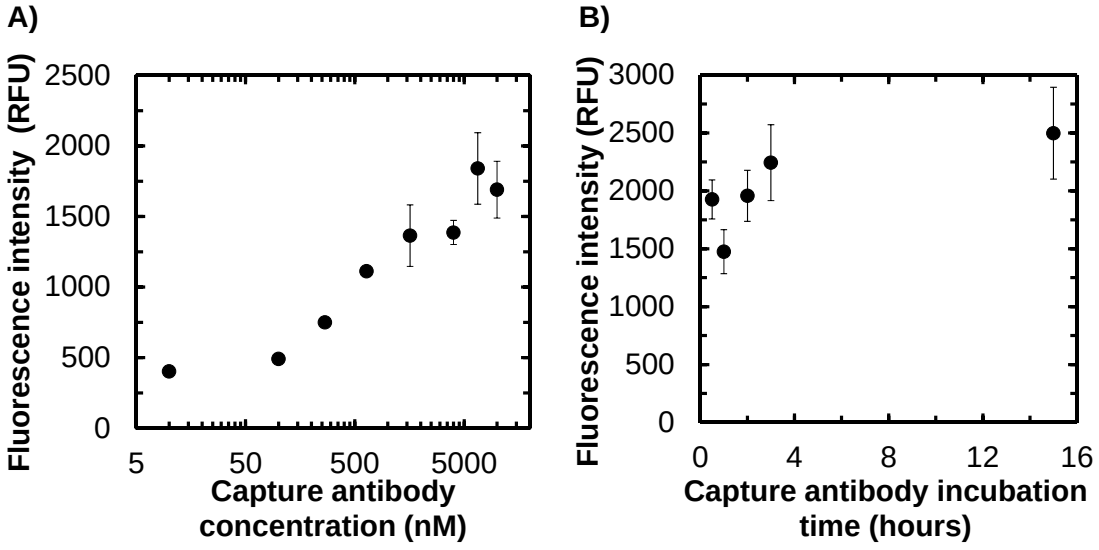
Supplementary Figure S5: UV-visible absorption spectrum of aqueous amplification solution with (dashed line) and without (solid black line) pH adjustment.



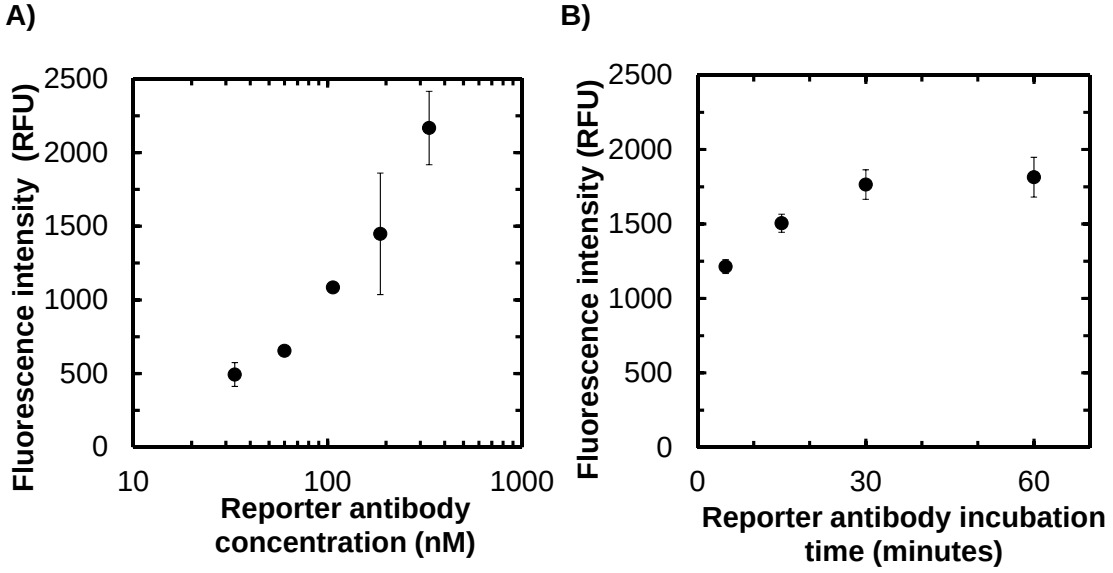
Supplementary Figure S6: Mean fluorescence intensity values for the detection of *Pf*HRP2 in a buffered solution on aldehyde-functionalized and unmodified Whatman No. 1 chromatography paper. The concentration (67 μM) and the incubation time (overnight) of the capture antibody, the concentration (130 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.



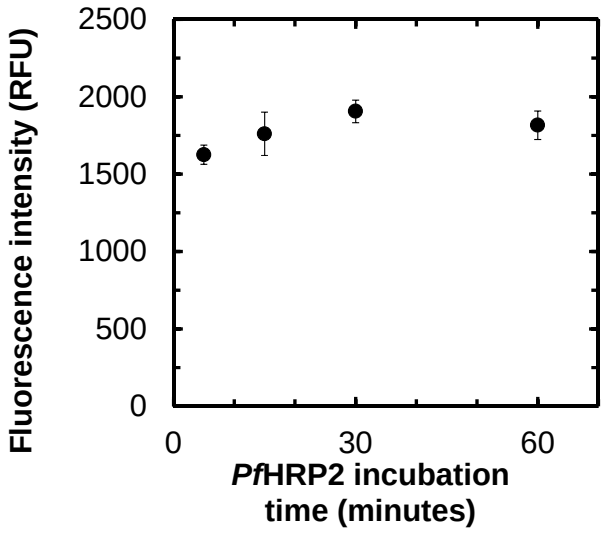
Supplementary Figure S7: Mean fluorescence intensity values for the detection of *Pf*HRP2 on paper in which the concentration and the incubation time of the capture antibody were varied. (A) Different concentrations of capture antibody were incubated overnight (15 hours). (B) 67 µM capture antibody was 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 PfHRP2 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 µM 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.



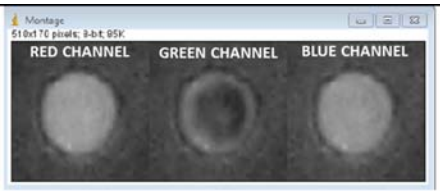
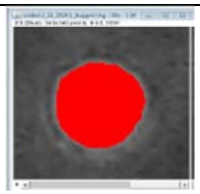
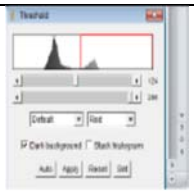
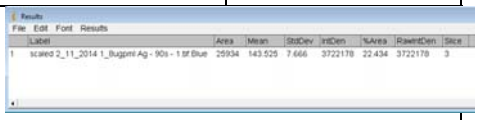
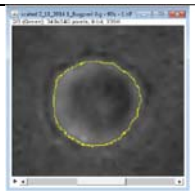
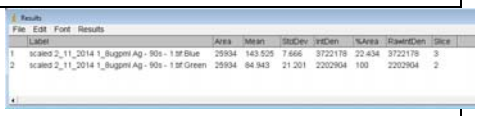
Supplementary Figure S8: Mean fluorescence intensity values for the detection of *Pf*HRP2 on paper in which the concentration and the incubation time of the eosin-conjugated reporter antibody were varied. The concentration (67 μM) and the incubation time (overnight) of the capture antibody and the concentration (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 μM 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±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 seen up to 30 minutes after which an increase in the incubation time led to a minimal increase in the fluorescent intensity.



Supplementary Figure S9: Mean fluorescence intensity values for the detection of *Pf*HRP2 on paper in which sample (containing *Pf*HRP2) incubation time was varied. 67 uM 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*	
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*	
7. Subtract the average intensity in the green channel from the average intensity in the blue channel		

Supplementary Table S2: Calculated colorimetric intensity values for each concentration of *Pf*HRP2 (n=8). The p-values shown in the table are calculated from a one-tailed unpaired t-test between the colorimetric intensity values of surfaces at the given concentration of *Pf*HRP2 and the colorimetric intensity values of surfaces tested with the preceding concentration of *Pf*HRP2.

Concentration of *Pf*HRP2 (nM)	Mean colorimetric intensity	Standard deviation	p-value (1-tailed unpaired t-test)
0	6.0	4.1	
1.3	3.5	2.4	0.0800
2.3	6.9	2.8	0.0111*
4.1	14.2	4.5	0.0011*
7.2	26.7	5.8	0.0002*
13	34.4	9.1	0.0334*
23	57.3	11.9	0.0004*
41	74.3	8.7	0.0032*
72	83.8	4.6	0.0105*
130	90.4	4.8	0.0071*

*p-value is less than 0.05 and indicates a statistically significant result

Supplementary Table S3: A comparison of polymerization-based amplification (PBA) with existing colorimetric methods for immunoassays on cellulose.

Colorimetric detection method	Contribution to total number of assay steps	Contribution to assay time*
Polymerization-based amplification	3 (Polymerization, rinse, addition of NaOH)	2-2.5 mins[#]
Enzymatic reactions	1 (Addition of substrate)	30 mins[5,9]
Gold nanoparticles with enhancement	1 (Addition of enhancement solution)	30 mins[9]

*All the studies referred to in this column used a flow-through system. Therefore, the contribution of the detection method to the overall assay time can be directly compared with our system.

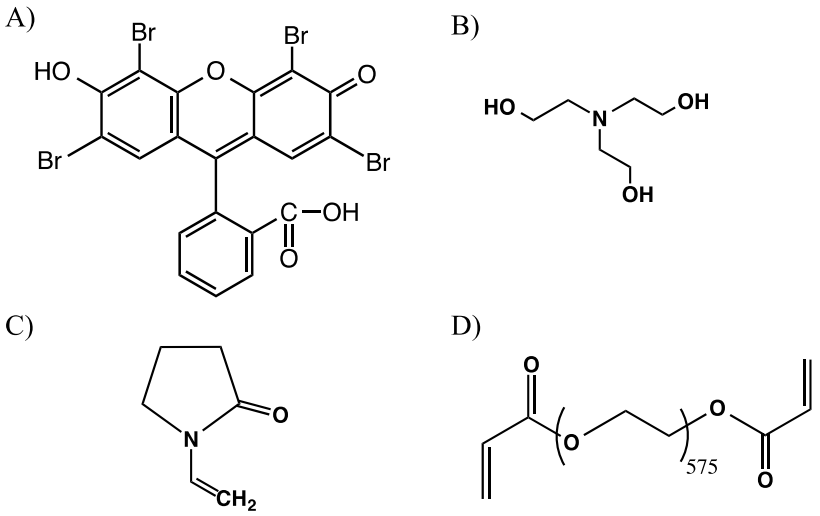
[#] This time includes all three PBA steps – polymerization (≤90 s), rinsing to remove unreacted solution (~30 - 60 s), and addition of NaOH (negligible; color change is instantaneous upon addition of NaOH).

Note: A color development time of 2-2.5 minutes compared to a time of 20-30 minutes has important implications. Because there is an end time beyond which the results of the enzymatic and gold nanoparticle-based tests cannot be trusted,[10] if a user walks away from the test (due to long color development times) and returns only a few minutes late, the test can become invalid (false positive). Users may be less likely to walk away in 2-2.5 minutes. Moreover, if an automated timer is used for PBA, such that the light automatically shuts off after a fixed time, the reaction will stop with the light and the user can come back and perform the rinse and the visualization step at a later time. The visualization itself is instantaneous upon addition of NaOH, and storage experiments showed no 'end' time (up to four months) after which the test became invalid.

SUPPLEMENTARY DISCUSSION

Eosin-mediated polymerization reaction and visualization of interfacial hydrogels on paper

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.[11–13] The eosin/tertiary amine photoinitiation system in particular has historically been used for the polymerization of acrylate monomers for encapsulation of cells.[14] When irradiated with green light, the excited triplet state of eosin (E*) accepts an electron from TEA. The TEA radical cation then loses a proton to generate the initiating TEA radical.[13,15,16] 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. The initiating radical or the radical terminus of a growing polymer chain can also react with the dissolved oxygen present in the system to generate less reactive peroxide radicals. The peroxide radicals are unreactive towards further propagation and

thus terminate the growth of the polymer chains.[17] Therefore, the free radical polymerization reactions in PBA were previously carried out under oxygen-free conditions by purging the system with an inert gas.[18] 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.[6] This observation was also used to develop a PBA system that is capable of generating interfacial polymers without purging the system.[4] The reaction kinetics of the eosin-based polymerization reaction in the presence of oxygen is still under active investigation and the reaction scheme presented in Figure DS2 consists of reactions that are thought to be the most important.

(A) Initiation

$$E \xrightarrow{h\nu} E^* \xrightarrow{TEA} (E-H)^\bullet + TEA^\bullet$$

(B) Propagation

$$TEA^\bullet + nM \longrightarrow TEA - M_n^\bullet$$

(C) Inhibition

$$TEA^\bullet + O_2 \longrightarrow TEA - OO^\bullet$$

$$TEA - M_n^\bullet + O_2 \longrightarrow TEA - M_nOO^\bullet$$

(D) Termination

$$TEA - M_n^\bullet + TEA - M_n^\bullet \longrightarrow TEA - M_{n+n} - TEA$$

$$TEA - M_n^\bullet + TEA - M_n^\bullet \longrightarrow TEA - M_n + TEA - M_n$$

(E) Eosin regeneration and termination

$$(E-H)^\bullet + TEA - OO^\bullet \text{ } '' \text{ } '' \rightarrow E + TEA - OO - H$$

$$TEA - M_nOO^\bullet + (E-H)^\bullet \text{ } '' \text{ } '' \rightarrow E + TEA - M_nOO - H$$

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.

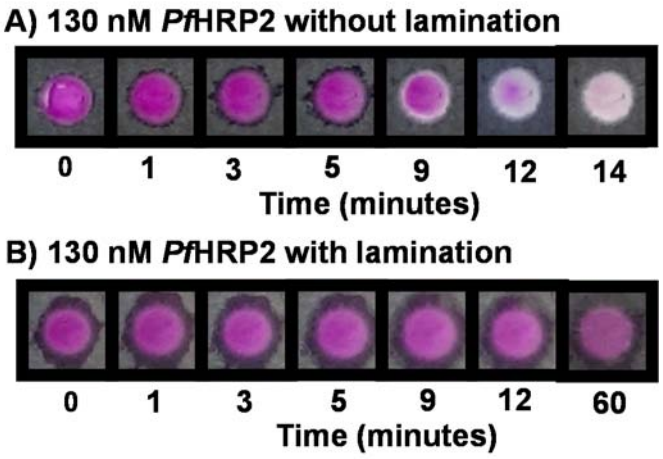
A) Paper surface with hydrogel

B) Paper surface without hydrogel

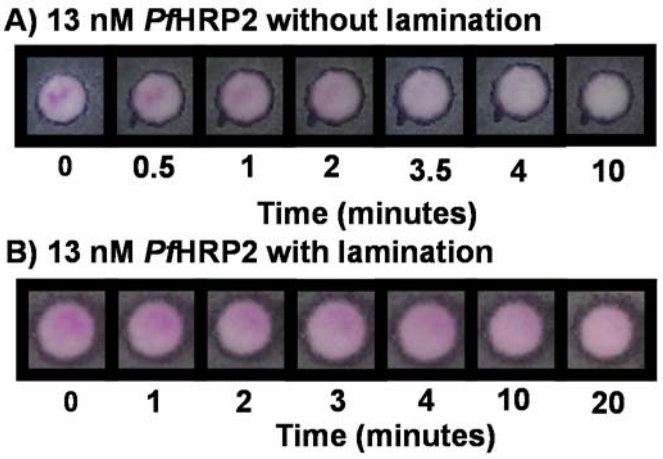
Supplementary Discussion Figure DS3: Visualization on paper (A) with hydrogel, and (B) without hydrogel, by swelling with a dye solution. The dye solution adheres non-specifically to paper, thus the colorimetric result is similar regardless of the presence of the hydrogel.

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 *Pf*HRP2 (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 *Pf*HRP2 (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 µL NaOH at t=0 minutes.

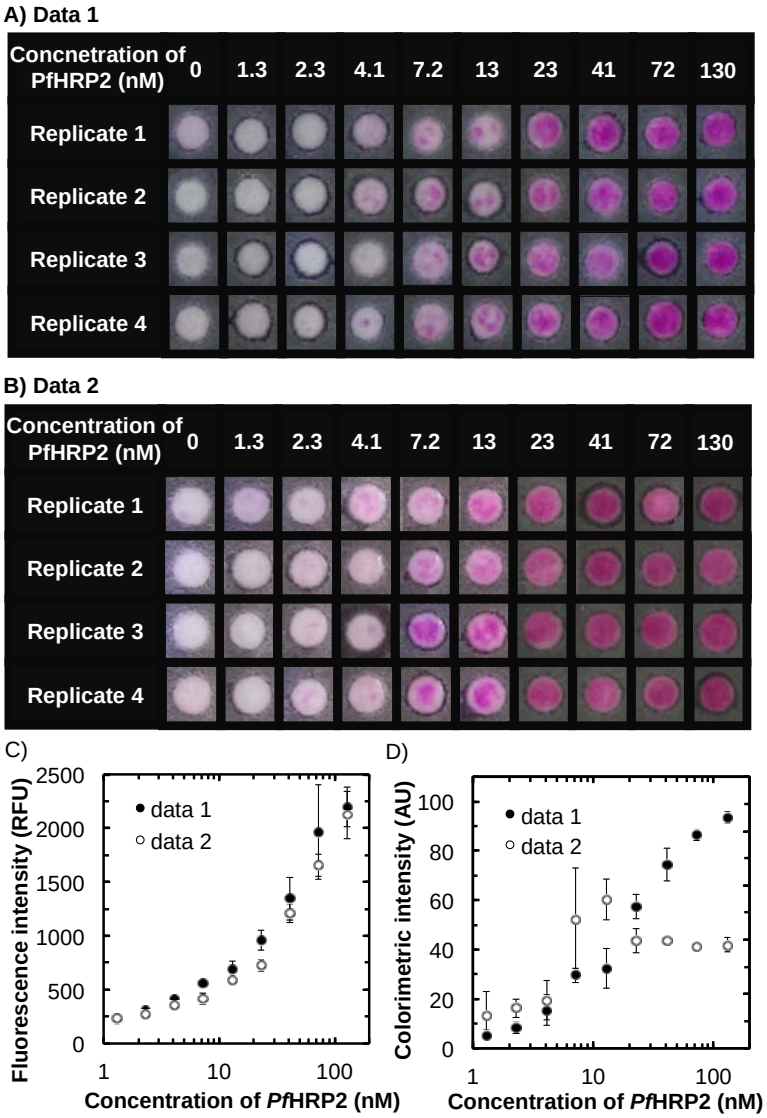


Supplementary Discussion Figure DS5: Images showing the colorimetric response for detection of 13 nM *Pf*HRP2 on paper (A) with, and (B) without lamination after addition of 2 µL NaOH at t=0 minutes.

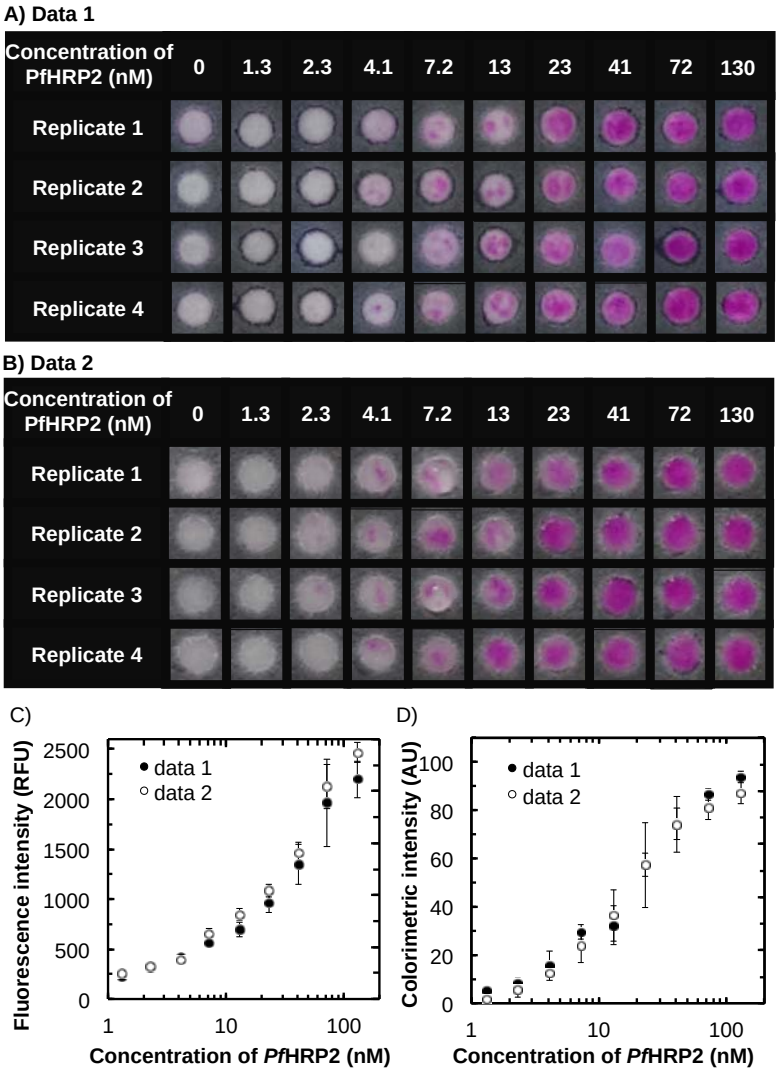
Effect of ambient light during imaging

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.

Paper surfaces that were prepared by overnight incubation of 67 μM 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 μL 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).



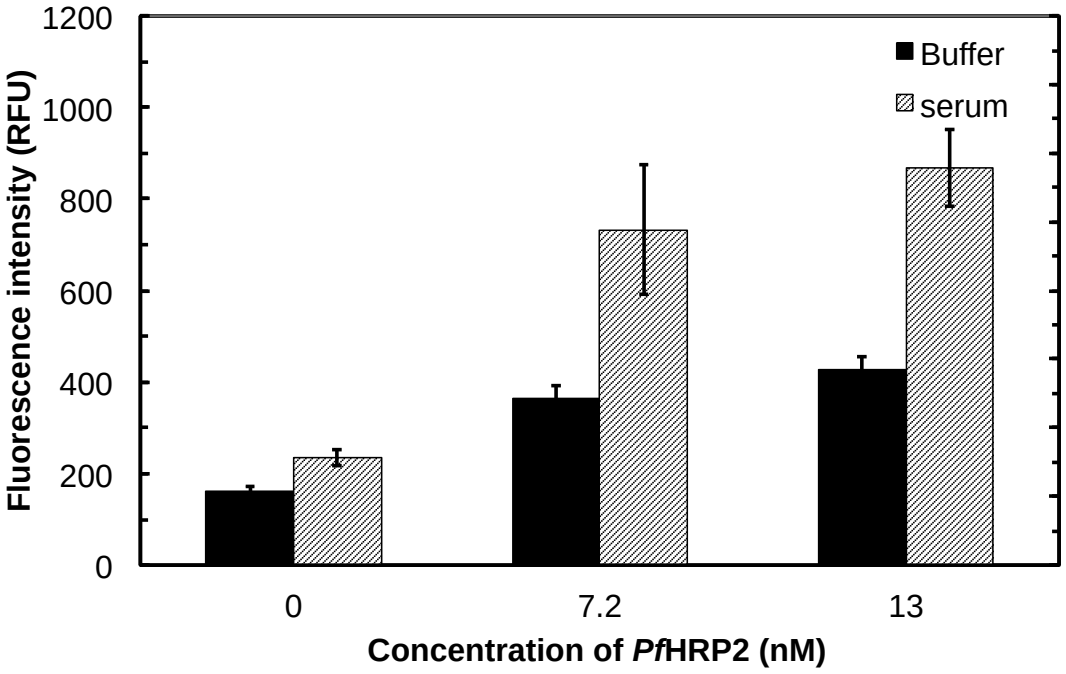
Supplementary Discussion Figure DS6: Images for a dose-response trial done on two different days, (A) and (B). The images in (A) were taken by illuminating the surfaces with an ordinary desk lamp and images in (B) were taken under ambient light. For (B), the surfaces with *Pf*HRP2 concentration of 0-13 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 *Pf*HRP2 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.

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. It was 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) because of higher non-specific binding of proteins in the serum to the capture antibody and/or the surface of the paper. 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. Therefore, the polymerization reaction is not sensitive to minor day-to-day variability.

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