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

SUPPLEMENTARY INFORMATION

Assessment of colorimetric amplification methods in a paper-based immunoassay for diagnosis of malaria

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1. MATERIALS

Whatman No. 1 chromatography paper, lyophilized bovine serum albumin (BSA), and glycerol were purchased from VWR (Radnor, PA, USA). Gel blot paper (GB 003, 15cm x 15 cm) was obtained from Whatman Inc. (Sanford, ME, USA). Sodium (meta) periodate ($NaIO_4$), poly(ethylene glycol) diacrylate (Mn=575) (PEGDA), triethanolamine (TEA), 1-vinyl-2-pyrrolidinone (VP), eosin Y disodium salt, dimethyl sulfoxide (DMSO), 10X phosphate buffered saline (PBS), phenolphthalein, hydrogen chloride, Tween® 20, pre-mixed BCIP®/NBT solution, and sterile-filtered US-origin human serum (from human male AB plasma) were obtained from Sigma Aldrich (St. Louis, MO, USA). 3,3'-diaminobenzidine (DAB) substrate tablets were purchased via VWR from Amresco Inc. (Solon, OH, USA). Tris (hydroxymethyl)aminomethane (Tris) and sodium chloride were purchased from Avantor Performance Materials (Center Valley, PA, USA). Ready-to-use 3,3',5,5' tetramethylbenzidine (TMB) substrate solution was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Eosin-5-isothiocyanate (EITC) was obtained from Marker Gene Technology (Eugene, OR, USA). Silver enhancer kit (light insensitive) was obtained from Abcam (Cambridge, MA, USA) and streptavidin – 20 nm gold conjugate (OD=3) was obtained from Cytodiagnotics Inc. (Burlington, ON, Canada). EZ-Link Sulfo-NHS-LC Biotin (8 x 1 mg ampules), BCA assay reagents, hydrogen peroxide and high-sensitivity streptavidin HRP conjugate was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Streptavidin and streptavidin alkaline phosphatase conjugate were obtained from Rockland Immunochemicals Inc. UltraCruz™ Micro G-25 Spin Columns were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, 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 capture antibody (ABMAL-04444, Clone 44) and monoclonal reporter antibody (ABMAL-0445, Clone 45) were purchased from Arista Biologicals Inc. (Allentown, PA, USA).

2. ADDITIONAL DESCRIPTION OF COLORIMETRIC DETECTION METHODS

2.1. Enzymatic reactions

We have used two common enzymatic labels, horseradish peroxidase (HRP) and alkaline phosphatase (ALP). For HRP, we used two different substrate solutions, i) a mixture of TMB and hydrogen peroxide, and ii) a mixture of DAB and hydrogen peroxide. TMB is a clear to pale-yellow colored compound that is oxidized by HRP to yield a cation free radical form that is blue in color and a diimine form that is yellow in color.[1] The resulting color can range between blue, green and yellow depending on the relative concentrations of the two oxidation products. DAB, on the other hand is oxidized by HRP to a free-radical form that polymerizes and forms a brown-colored precipitate on the surface.[2] For ALP, we used a mixture of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). ALP catalyzes the formation of an 'indoxyl' intermediate from BCIP that reacts with NBT to produce a visible 'diformazan' product.[3]

2.2. Gold nanoparticles and silver amplification

Gold nanoparticles are one of the most widely used colorimetric agents for commercial point-of-care (POC) diagnostic tests, such as lateral flow immunoassays.[4] Lateral flow immunoassays are typically done on nitrocellulose membranes and on these surfaces, gold nanoparticles can produce a visible band of color by aggregating at a test line of capture antibodies in the presence of analyte of interest.[5]. An additional silver amplification method has been reported for visualization using gold nanoparticles on cellulose surfaces.[6] Gold nanoparticles present on the surface are thought to act as nucleation sites for formation of silver clusters around gold nanoparticles followed by a growth step catalyzed by the precipitated silver.[7] The deposition of silver is dependent on time of contact of the silver enhancement solution with the surface and it is well-established that silver can self-nucleate after some time.

2.3. Polymerization-based amplification (PBA)

PBA utilizes photoinitiators as a label on biomolecules and uses the formation of a visible interfacial hydrogel as a sensing mechanism.[8–12] Eosin can be used as a photoinitiator and when a surface containing eosin is contacted with an aqueous solution containing acrylate monomers and a coinitiator, triethanolamine, and illuminated with visible light, a free radical polymerization reaction occurs and results in the formation of an interfacial

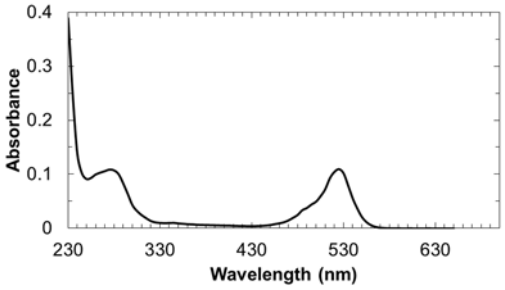
hydrogel. The eosin/tertiary amine initiation system has been modified by addition of submicromolar concentration of free eosin into the aqueous solution to overcome oxygen inhibition such that it can be carried out in air.[11] This PBA system has also been adapted for use with paper-based immunoassays by incorporating a pH indicator, phenolphthalein, for visualization.[12] In brief, for paper-based assays, the pH of the aqueous solution is adjusted to just below 8 in order to keep phenolphthalein in its colorless form to prevent interference with the absorption of light by eosin. As the polymerization occurs, a hydrogel is formed on the surface and phenolphthalein gets trapped in the pores of the hydrogel. The unpolymerized solution is then washed away with water. If a hydrogel containing trapped phenolphthalein is present on the surface, phenolphthalein turns bright pink on addition of a basic solution and can be easily seen by the unaided eye. As previously shown,[12] the colorimetric sensing using PBA can be divided into two distinct steps, i) photo-polymerization reaction, and ii) visualization. The result of the photo-polymerization step is dependent on the illumination time, which is a variable that is fixed during the design phase of the assay and is automated so that it is not at the discretion of the user. The addition of basic solution after polymerization is the user-controlled step during sensing.

3. ADDITIONAL EXPERIMENTAL DETAILS

3.1. Preparation of eosin-conjugated streptavidin and eosin-conjugated reporter antibody

The method of conjugation of EITC to proteins has been described previously.[9,11,12] EITC (1 mg) was dissolved in 100 μL of DMSO to prepare a 10 mg/mL EITC stock solution. To prepare the streptavidin conjugate, 10 μL of the EITC stock solution was mixed with a 100 μL solution of streptavidin (1 mg/mL) in 0.1 M sodium bicarbonate buffer (pH 9.0) to give a total reaction volume of 110 μL and the reaction mixture was protected from light and placed at 4 °C overnight. To prepare the conjugate with the reporter antibody, 20 μL of the EITC stock solution was mixed with 400 μL of the reporter antibody solution (5.3 mg/mL) in 0.1 M bicarbonate buffer solution to give a total reaction volume of 420 μL and the reaction was allowed to occur at 4 °C for 5 hours. During the reactions, the isothiocyanate functional group of EITC reacts with the amine group of the lysine residues of the proteins to form a thiourea bond. At the end of the reaction, the excess EITC was separated from the eosin-conjugated streptavidin and eosin-conjugated reporter antibody by size-exclusion chromatography with Sephadex matrix (Micro G-25 Spin-Column).

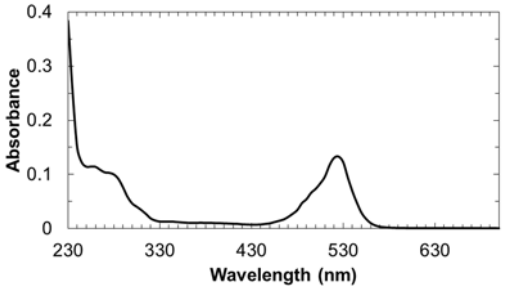
UV–visible absorbance spectroscopy was used to determine the concentration of the protein and the average number of eosin molecules coupled to each streptavidin molecule (Figure S1) or each reporter antibody molecule (Figure S2) by taking an absorbance scan of the purified conjugate. The purified and characterized conjugates were diluted to make 50% v/v glycerol stock and stored at -20 °C until use.



Supplementary Figure S1: UV-visible absorption spectrum of EITC conjugated to streptavidin (2.8 EITC molecules per streptavidin molecule). For determining the average number of eosin molecules coupled to each streptavidin molecule, the following equation was used:

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

where $n_{EITC}$is the number of molecules of eosin, $n_{SA}$is the number of molecules of SA, $Abs_{280}$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 $\varepsilon_{SA,280}$ = 173,000 $M^{-1}cm^{-1}$.



Supplementary Figure S2: UV-visible absorption spectrum of EITC conjugated to reporter antibody (7 EITC molecules per IgG molecule). For determining the average number of eosin molecules coupled to each IgG molecule, the following equation was used:

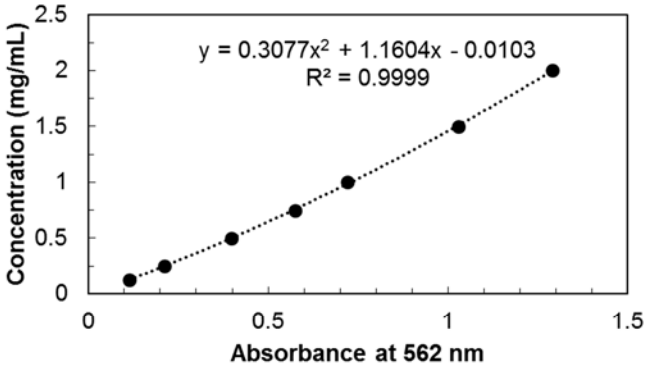
$$n_{EITC}/n_{IgG} = \left(Abs_{525}/\varepsilon_{EITC,525}\right)/\left[\left\{Abs_{280} - \left(Abs_{525}\varepsilon_{EITC,280}/\varepsilon_{EITC,525}\right)\right\}/\varepsilon_{IgG,280}\right]$$

where $n_{EITC}$is the number of molecules of eosin, $n_{IgG}$is the number of molecules of IgG, $Abs_{280}$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 $\varepsilon_{IgG,280}$ = 280,200 $M^{-1}cm^{-1}$.

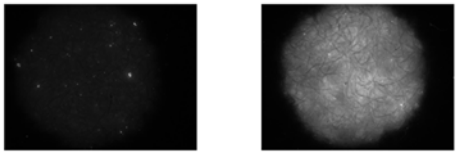
3.2. Preparation of biotin-conjugated reporter antibody

Biotin-conjugated reporter antibody was prepared through reaction of the reporter antibody with the EZ-Link Sulfo-NHS LC biotin reagent. 190 μL of 5.57 mg mL^{-1} reporter antibody in 1X PBS was mixed with 14.2 μL of 10 mM biotin reagent (20-fold molar excess) and allowed to react for 2 hours on ice. During the reaction, the sulfo-NHS ester group reacts with the primary amine groups available on the lysine residues of the antibody to form an amide bond, thereby covalently attaching biotin to the protein molecules.[13] At the end of the reaction, the excess biotin was separated from the biotin-conjugated antibody by size-exclusion chromatography with Sephadex matrix (Micro G-25 Spin-Column). The antibody concentration in the purified conjugate was determined to be 3.5 mg mL^{-1} using the BCA assay. Figure S3A shows the standard curve obtained in the BCA assay. The presence of biotin on the reporter antibody was verified by performing an immunoassay for detection of *Pf*HRP2 with the purified conjugate, followed by streptavidin-eosin and measuring the fluorescence of eosin on the surface 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. (Figure S3B).

A) Standard curve for the BCA assay for antibody concentration determination



B) Using fluorescence to detect the activity of the biotinylated reporter antibody

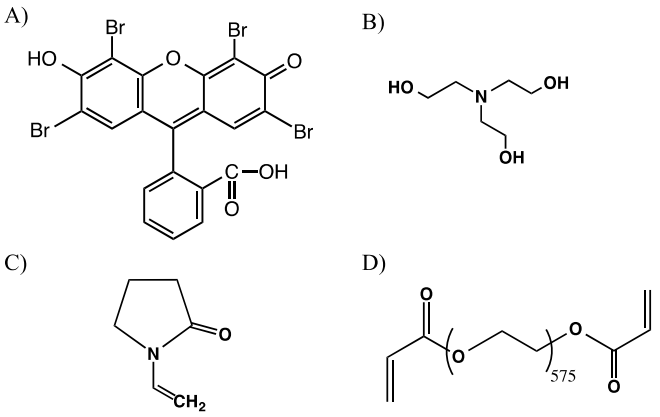


Negative control

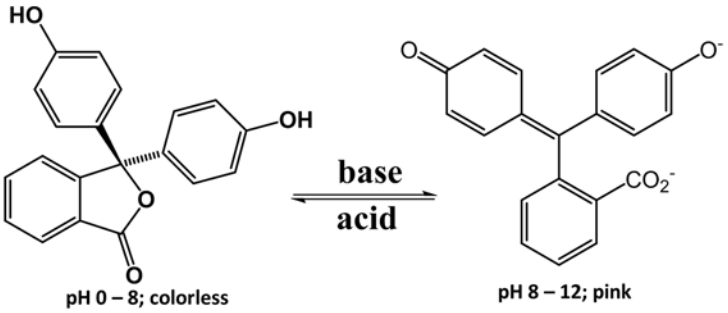
Positive control

Supplementary Figure S3: Preparation and characterization of biotinylated reporter antibody. (A) The concentration of the biotinylated-reporter conjugate was determined using the BCA assay for protein concentration determination. Albumin standard with a known concentration of 2 mg mL^{-1} was serially diluted to obtain solutions containing 1.5, 1.0, 0.75, 0.5, 0.25, and 0.125 mg mL^{-1} solutions. The standard solutions and the conjugated reporter antibody solution were reacted with the BCA reagents and a standard curve was obtained using the albumin samples. The absorbance of the conjugated antibody was compared to the standard curve, to obtain the concentration of the reporter antibody solution. (B) Fluorescence from eosin was used to determine the functionality of the biotinylated antibody in a surface immunoassay. Paper surfaces containing the capture antibody were contacted with 10 μg mL^{-1} *Pf*HRP2 in serum (positive control) and undiluted serum (negative control). These surfaces were then contacted with 50 μg mL^{-1} purified antibody solution prepared above, followed by 0.2 μM SA-eosin. The surfaces were then imaged with a fluorescence microscope. The presence of a higher fluorescence signal for positive control compared to the negative control indicates that the purified reporter antibody is active, i.e., capable of binding *Pf*HRP2 on the surface and is conjugated to biotin.

3.3. Reagents used in polymerization-based amplification

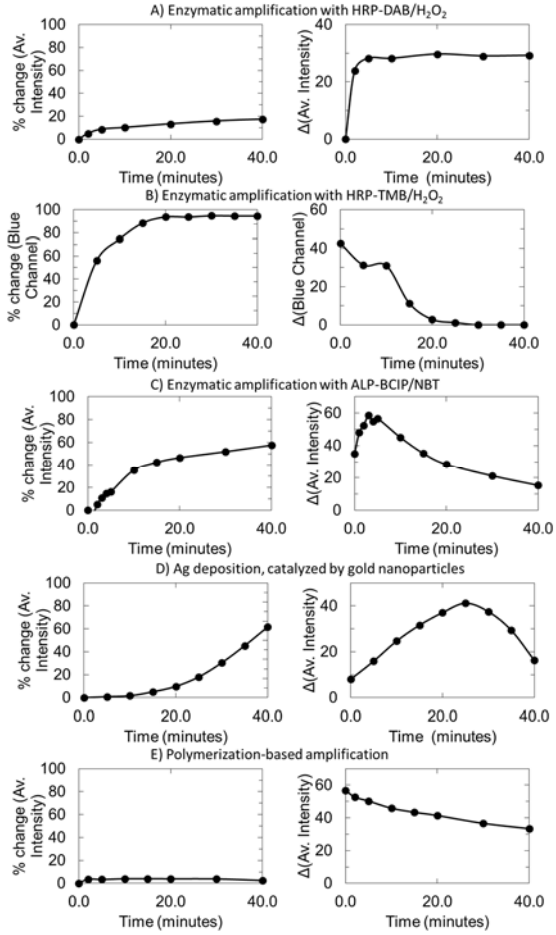


Supplementary Figure S4: Chemical structures of species used in photo-polymerization-based amplification (PBA) reaction (A) eosin Y, a photoinitiator, (B) triethanolamine, a coinitiator (C) 1-vinyl-2-pyrrolidinone, an accelerating agent, and (C) poly(ethylene glycol diacrylate, a cross-linking agent



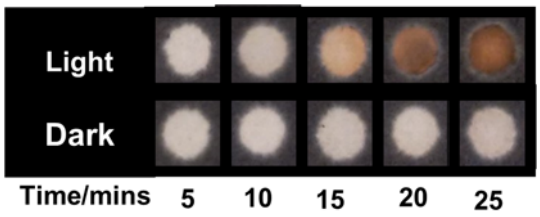
Supplementary Figure S5: Equilibrium between colorless and pink isomer of phenolphthalein, the visualization agent used in PBA, as a function of pH.

3.4. Quantification of the intensity of positive and negative test surfaces with time



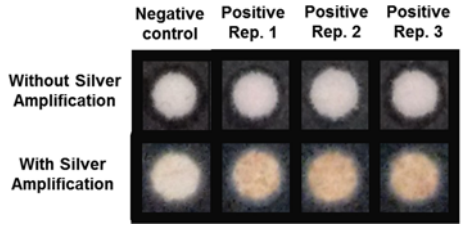
Supplementary Figure S6: Quantifying the percentage change in the colorimetric intensity of the negative controls with time (LHS panel) and the difference in intensities between the positive surfaces and negative surfaces (RHS panel) for the colorimetric methods shown in Figure 1 of the paper. (A) Enzymatic amplification using HRP with DAB/H_2O_2 substrate system, (B) enzymatic amplification using HRP with TMB/H_2O_2 substrate system, (C) Enzymatic amplification using ALP with BCIP/NBT substrate system, (D) silver deposition on gold nanoparticles, and (E) polymerization based amplification visualized with phenolphthalein. The average intensity is calculated as the square root of the sum of squares of intensities of all three channels – red, green and blue. For HRP-TMB/H_2O_2, only the intensity of the blue channel is used because using average intensity to calculate the difference in RHS panel leads to negative values. The percentage change is calculated based on the value of the intensity at t=0 seconds.

3.5. Effect of exposure to ambient indoor light on self-nucleation of silver



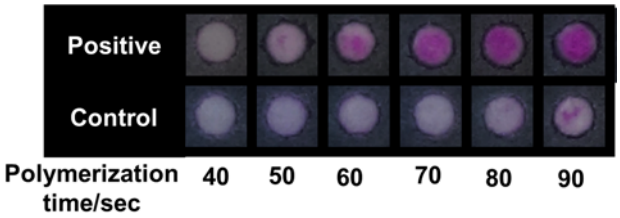
Supplementary Figure S7: The kinetics of self-nucleation of silver from the silver enhancement solution become faster if the solution is exposed to indoor light (row labeled with 'light') compared to the situation when the silver enhancement solution is protected from light (row labeled as 'dark'). For this experiment, the silver enhancement solution was made by mixing the initiator and the enhancer and placed on paper surfaces for different times on a bench in the laboratory (light) or in a closed drawer (dark). After the specified time had elapsed, the surfaces were rinsed with water, allowed to dry and imaged.

3.6. Surfaces tested with gold nanoparticles, with and without silver amplification



Supplementary Figure S8: Positive (130 nM *Pf*HRP2 in serum) and negative surfaces (serum) were tested using 50 μg mL^{-1} biotinylated reporter antibody followed by streptavidin-20 nm AuNP conjugate at OD=0.6. The surfaces were rinsed with water and imaged. The same surfaces were contacted with the silver amplification solution for 25 minutes in dark, rinsed, allowed to dry, and imaged again. The results show that silver amplification was necessary to generate enough contrast to visually differentiate the positive surfaces from the negative surfaces. AuNPs at OD=0.6, by themselves did not produce a visible color on the paper surface.

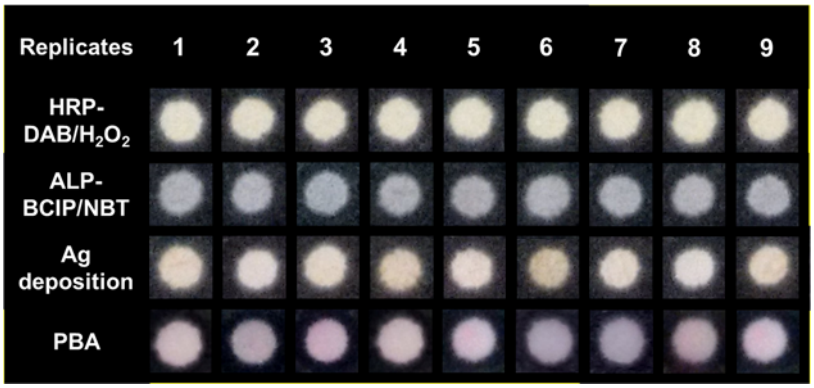
3.7. Importance of illumination time in PBA



Supplementary Figure S9: The result of polymerization-based amplification depends on the time of illumination of the surface. Positive and negative surfaces were prepared and illuminated for different times and the results were visualized by adding a basic solution. The illumination time, once determined for an assay in a given matrix, remains the same from day-to-day for the same illumination conditions and monomer.[12] These images were taken with the camera application in an HTC One Mini.

3.8. Replicates for the dilution series

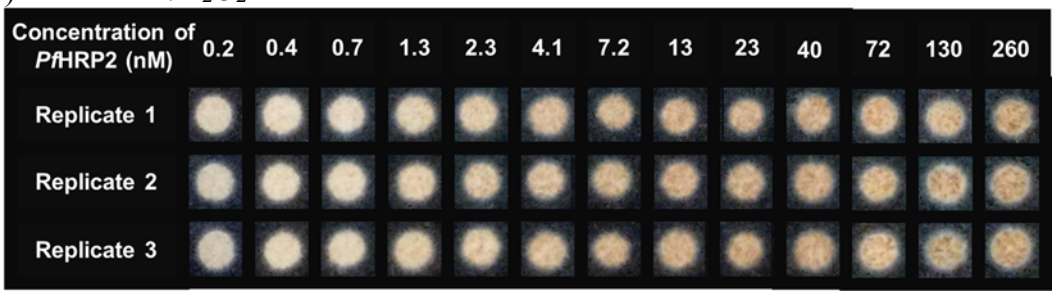
3.8.1. Negative control replicates



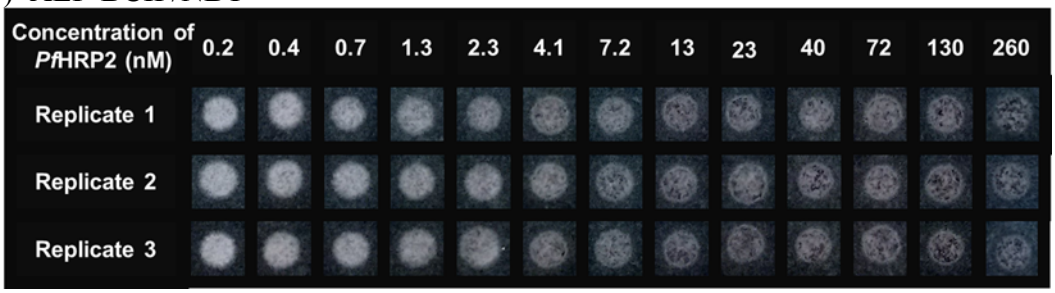
Supplementary Figure S10: Replicates of the negative controls in the dilution series experiments for the four different colorimetric methods show in Figure 2. All images were taken with Moto E2.

3.8.2. Positive replicates

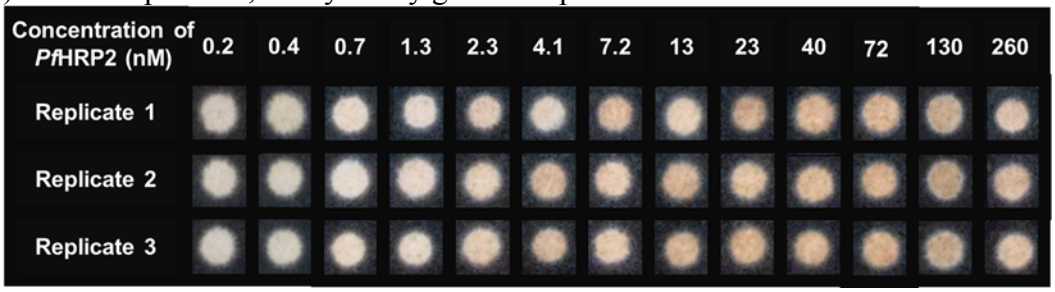
A) HRP-DAB/H_2O_2



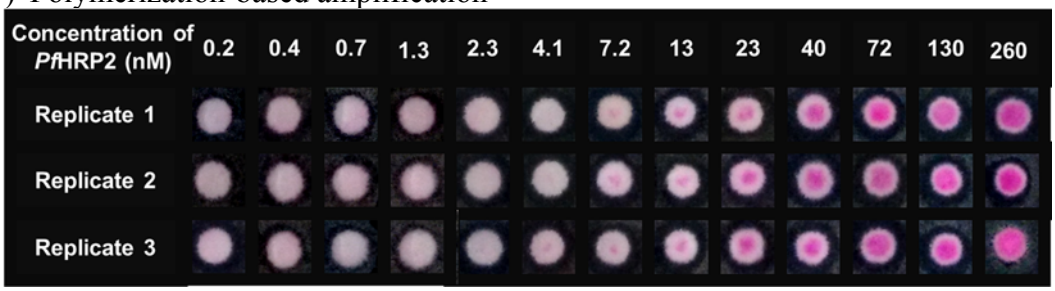
B) ALP-BCIP/NBT



C) Silver deposition, catalyzed by gold nanoparticles



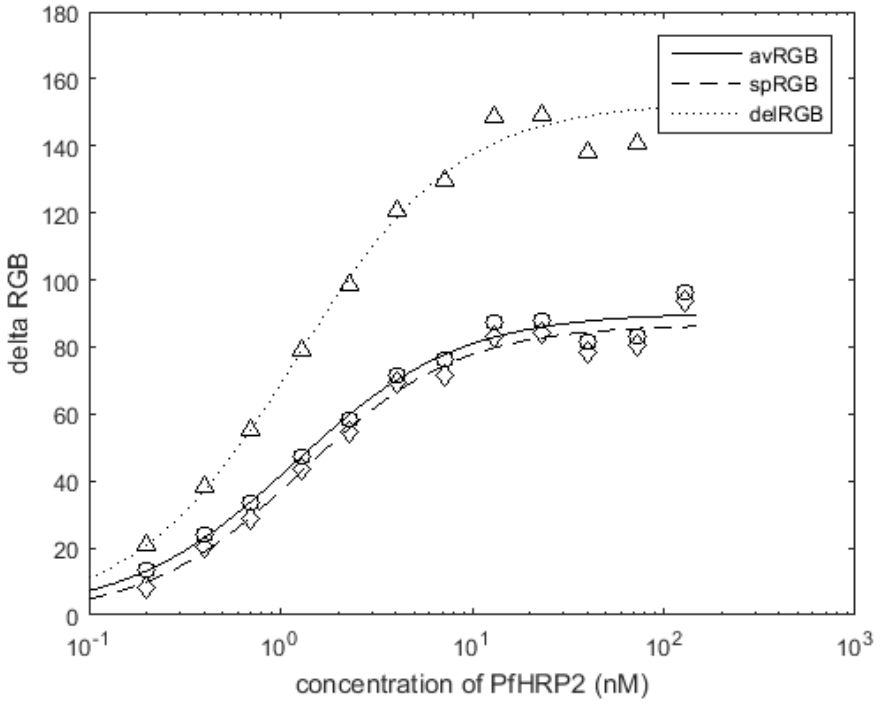
D) Polymerization-based amplification



Supplementary Figure S11: Replicates of positive controls in the dilution series experiments across the full range of concentrations of *Pf*HRP2 that were tested, 0.2 nM – 260 nM for A) enzymatic amplification with HRP using DAB/H_2O_2 solution as the substrate, B) enzymatic amplification with ALP using BCIP/NBT solution as substrate, C) using silver deposition, catalyzed by gold nanoparticles, and D) polymerization-based amplification.

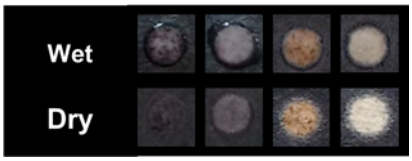
3.9. Factors affecting imaging and quantitative analysis

Cellphone cameras capture images in the RGB color space. An RGB image can be separated into individual red, green, and blue channels and the intensity of the test zone can be computed for each of the three channels. Once these intensities have been calculated, there are different ways of analyzing the numbers obtained depending on the hue of the color being analyzed.[14] Depending on the method chosen, the absolute values can differ dramatically. As an example, analysis of the dilution array for ALP-BCIP/NBT results is shown with the background subtracted RGB intensity calculated in three different ways (Figure S11). If R, G, and B are the intensities of the red, green, and blue channels of the positive surfaces and R_0, G_0, and B_0 are the intensities of the red, green and blue channels of the control surfaces, respectively, then three different methods are, i) difference in the average intensity of Blue channel between the positive and the control surfaces (spRGB), i.e., $B-B_0$, ii) average intensity of all three channels of negative surfaces subtracted from the average intensity of all three channels of positive surfaces (avRGB), i.e. , $\sqrt{R^2+G^2+B^2}-\sqrt{R_0^2+G_0^2+B_0^2}$ and iii) average intensity according to Murdock et al.(delRGB)[6], i.e., $\sqrt{(R-R_0)^2+(G-G_0)^2+(B-B_0)^2}$. The analysis and the sigmoidal fit to the data show that depending on the method chosen to calculate the intensities, the overall trend remains the same but the absolute values can differ greatly.



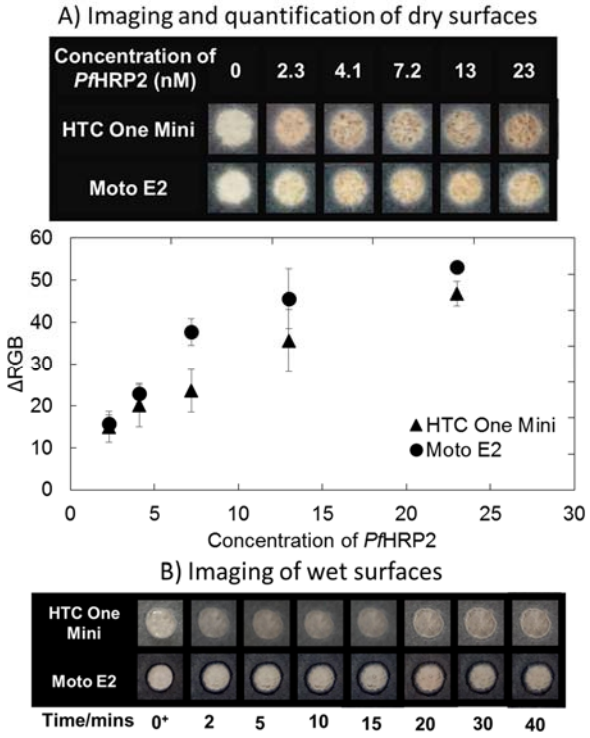
Supplementary Figure S12: Using three different methods to report the change in intensity in RGB color space for surfaces tested with ALP-BCIP/NBT, i) average intensity of the blue channel (spRGB), ii) average intensity of all three channels (avRGB), and iii) average intensity according to Murdock et al. (delRGB). The data points show the calculated values and the lines show the sigmoidal fits.

3.9.1. Imaging wet versus dry



Supplementary Figure S13: The effect of surface condition on the appearance of color. Same surfaces were imaged while they were wet and when they became dry. The appearance of color on the surface is different for the two cases and also affects the absolute values of the red, green and blue channel intensities in the captured image.

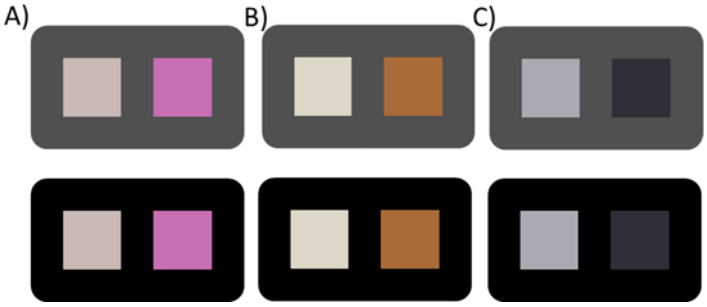
3.9.2. Dependence on device



Supplementary Figure S14: Effect of device on images. (A) The images of the same dry surfaces obtained using HRP-DAB/H_2O_2 taken with two different smartphones. On changing the device, the qualitative trend in images remains the same, but the absolute values of the red, green, and blue channels can be very different. (B) The wet negative control surfaces for HRP-DAB/H_2O_2 taken with two different smartphones appear very different.

3.9.3. Same ΔCIE values for different colors

$$\Delta CIE \approx 50$$



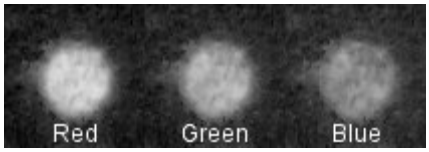
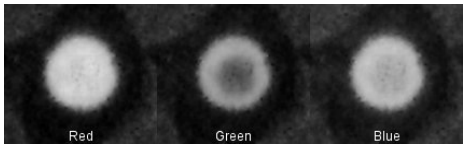
Supplementary Figure S15: The values of red, green and blue channel intensities were taken from the images of the positive and negative surfaces in Figure 2 for three different colors seen in experiments, (A) for PBA, (B) for HRP-DAB/H_2O_2 and silver amplification, and (C) for ALP-BCIP/NBT and used to simulate these colors in MS PowerPoint. The range of the red, green and blue channel intensities for each of the color transitions was extended and also converted to ΔCIE values using the MATLAB function 'rgb2lab'. This simulated data shows that a value of ΔCIE=50 is theoretically possible for all three color transitions that were observed experimentally. The two rows show the negative and positive samples generated in MS PowerPoint using the values of red, green and blue channel intensities that when converted to CIELAB color space have a ΔCIE value of 50. The RGB values are identical in the first and the second row and only the background color is changed from gray to black showing that the perception of the same color can be affected by the background color of the wax on the paper.

3.10. Quantification of colored images

Steps for obtaining red, green and blue channel intensities from the images captured with the cellphone were as follows:

1) Open the image in ImageJ
2) Separate the image into red, green, and blue channels
3) Select the test zone
4) Measure the average intensity of each of the three channels
5) Export the measured intensities into excel for further processing.
6) The individual intensities were then used to calculate the ΔRGB values according to the color being analyzed, as discussed in the methods section.

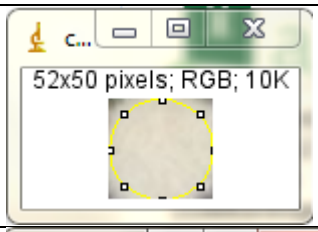
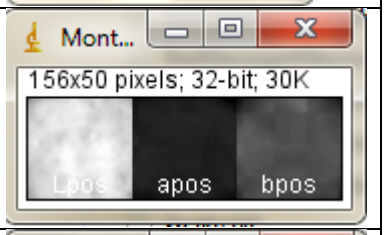
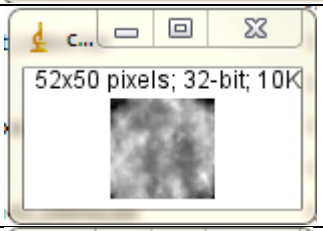
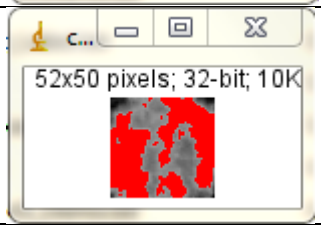
Supplementary Table S1: Positive controls separated into red, green and blue channels

Colorimetric method	Captured RGB Image	Image separated into Red, Green and Blue channels
1. HRP-DAB/H_2O_2 – positive control		
2. ALP-BCIP/NBT – positive control		
3. Silver amplification – positive control		
4. PBA – positive control		

Steps for obtaining ΔCIE values from the images captured with the cellphone were as follows:

1) The captured RGB images for a positive and negative control were cropped to the same size so that the test zones would overlap for the following steps.
2) Both the images were converted to CIELAB color space using the 'Colour Transform' function in ImageJ. The converted images were separated into L, a, and b channels and labelled L, a, and b for the positive control and L_0, a_0, and b_0 for the negative controls.
3) ImageJ was used to perform arithmetic operations, to obtain a final image such that the pixel values corresponded to $\Delta CIE = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2}$
4) The average of the highest ΔCIE values from the test zone was chosen as the representative ΔCIE value. An example of the above steps using positive and negative control images for surfaces tested with HRP-DAB/H2O2 is shown in Table S2.

Supplementary Table S2: An example calculation of ΔCIE values from images of positive and negative surfaces captured with a cellphone

Step	Type of well	Image
1. Crop the RGB images to the same size to allow the test zones to overlap	Positive	 pos
	Negative	
2. Convert the images to CIELA color space	Positive	
	Negative	
3. Perform arithmetic operations to obtain a final image where pixel values correspond to ΔCIE values	-NA-	
4.Pick the appropriate ΔCIE values from the final image	-NA-	

3.11. Evaluating the cost of assays

Supplementary Table S3: Cost contributions in different colorimetric methods

Step	Total cost per assay* ($)
Paper preparation	0.0034
Binding reactions (using biotinylated reporter)	0.1680
PBA (SA-EITC as label)	0.0050
ALP-BCIP/NBT (SA-ALP as label)	0.0142
HRP-DAB/H_2O_2 (SA-HRP as label)	0.0102
HRP – TMB/H_2O_2 (SA-HRP as label)	0.0155
Silver amplification (SA-AuNPs as label)	0.4576

Supplementary Table S4: Breakdown of cost components of each step from Table S3

Type	Reagent	Price**	Amount per assay	Cost per assay ($)
Binding reactions	Capture antibody	$55 per mg	2 µg	0.1100
	Reporter antibody	$55 per mg	0.25 µg	0.0153
	Biotin-NHS	$17.09 per mg	2.5 µg	0.0427
Paper preparation	Chromatography paper	$0.4855 per sheet (20 cm X 20 cm)	1 well (300 wells/sheet)	0.0016
	$NaIO_4$	$0.677 per g	1.43 mg/well	0.0010
	Wax	$0.2583 per page	1/300 of a page	0.0009
PBA	PEGDA	$0.1856 per mL	0.002 mL	3.7E-4
	TEA	$0.01855 per mL	0.0004 mL	7.42E-6
	VP	$0.07627 per mL	0.0002 mL	1.53E-5
	Eosin Y	$5.06 per g	0.007 µg	3.54E-8
	HCl	$1.5313 per mole	0.4 µmoles	6.13E-7
	Phenolphthalein	$0.256 per g	10 µg	2.56E-6
	NaOH	$0.0718 per g	40 µg	2.87E-6
	Streptavidin	$40.22 per mg	0.1 µg	0.0040
	EITC	$2.0728 per mg	0.3 µg	6.2E-4
ALP	SA-ALP	$170.73 per mg	0.05 µg	8.54E-3
	BCIP/NBT	$0.565 per mL	10 µL	0.00565
Silver amplification	SA-AuNPs	$225 per mL (OD=3)	10 µL (OD=0.6)	0.45
	Silver amplification	$0.764 per mL	10 µL	7.64E-3
HRP	SA-HRP	$175.21 per mg	0.05 µg	0.00876
	DAB	$0.13934 per mL	10 µL	0.001393
	TMB	$0.6785 per mL	10 µL	0.006785

**The prices of reagents were taken to be the minimum listed on the vendor sites. The prices are for laboratory test and effects of scaling up have not been considered.

3.12. Summary of the results

Supplementary Table S5: A comparison of the characteristics of colorimetric methods used in this study

Colorimetric detection method	Contribution to total number of assay steps	Waiting time till optimum readout*	Significant Change in appearance of negative control after optimal time	Contribution to total assay time
Polymerization-based amplification	3 (Polymerization, rinse, add NaOH)	-none-	No	2-2.5 mins[#]
Enzymatic reactions (HRP-DAB/H_2O_2)	2 (Addition of substrate, rinse)	8 mins	No	≥ 5 mins[**]
Enzymatic reactions (ALP-BCIP/NBT)	2 (Addition of substrate, rinse)	4 mins	Yes	≥ 3 mins[**]
Silver deposition	2 (Addition of silver enhancement solution, rinse)	25 mins	Yes	≥ 20 mins[**]

*As estimated from Figure 1 and Supplementary Figure S6. These are the times used in the dilution series in our study. These times can vary depending on the concentrations of the labels used in the assay.

#This time includes all three steps – polymerization (80 s), rinsing to remove unpolymerized solution (~30 - 60 s), and addition of NaOH (negligible; color change is instantaneous upon addition of NaOH)

**These times are the earliest we could reliably detect the color change by eye for positive surfaces tested with high concentrations of *Pf*HRP2 in our assays.

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