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ELECTRONIC SUPPORTING INFORMATION

Direct comparison of colorimetric signal amplification techniques in lateral flow immunoassays

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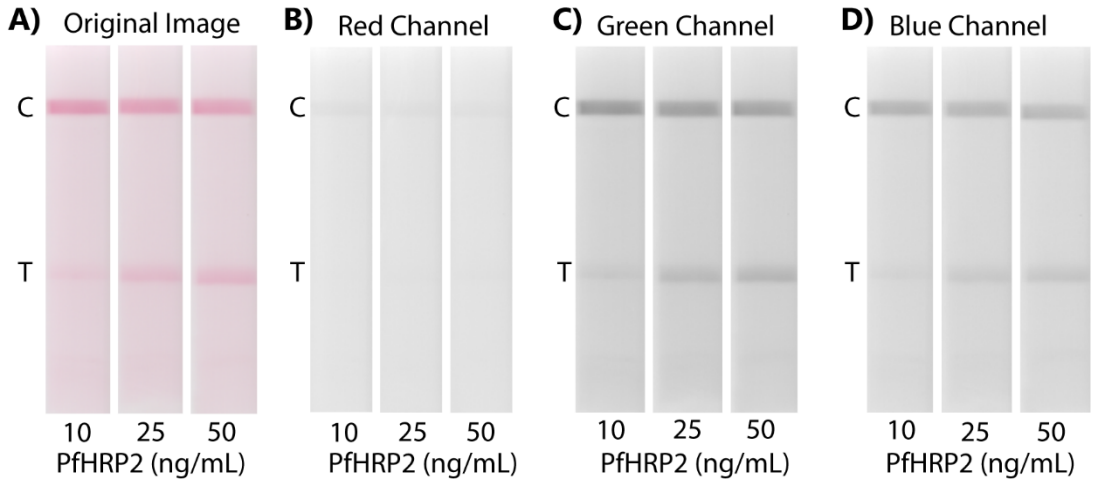
A. Image acquisition and RGB analysis

Any colour can be represented by a combination of red, green and blue, and since we had a white background, the colours red, green, and blue in RGB can be represented as (255,0,0), (0,255,0) and (0,0,255), respectively. For enhancing the range of pixel intensity variation, images were split into three colour channels – red, blue, and green, and a single-colour channel was used for analysis.



E)

PfHRP2 (ng/mL)	Test line intensity			
	Pink	Red	Green	Blue
$T_1 = 10$	40.376	27.524	50.886	41.616
$T_2 = 25$	47.421	30.267	60.462	48.653
$T_3 = 50$	48.492	30.442	62.81	50.825

F)

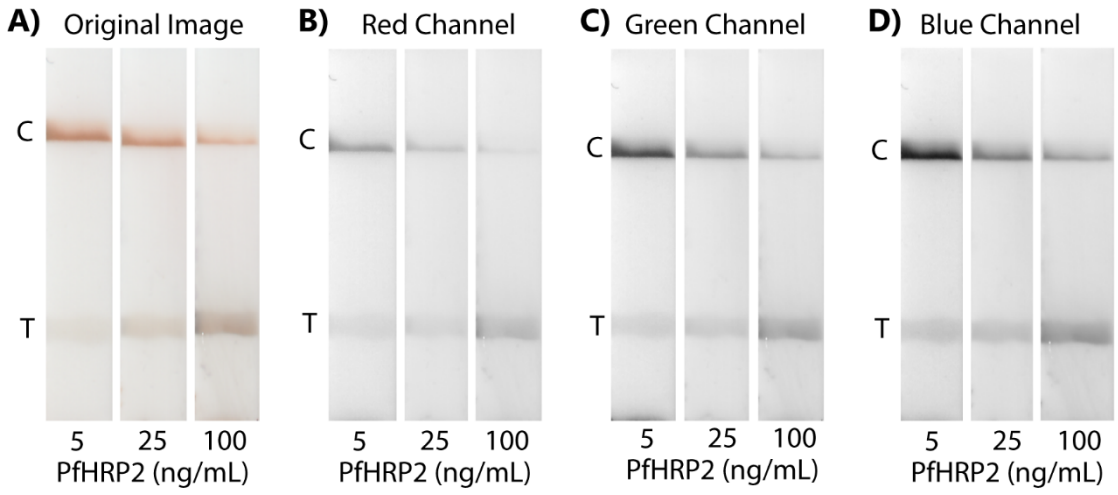
PfHRP2	Ratio of test line intensity			
	Pink	Red	Green	Blue
—	—	—	—	—
T_1 / T_2	0.8514	0.9093	0.8416	0.8553
T_2 / T_3	0.9779	0.9942	0.9626	0.9572

Figure S1 Image analysis of test and control line intensities for gold nanoparticle assay using ImageJ (A-F).

A-D. Images showing the variation of test line intensity for three different PfHRP2 concentrations (10,25, and 50 ng/mL) for original image (pink), red, green and blue channel. E-F. The corresponding test line intensities and their ratio for three different hCG concentrations (10,25, and 50 ng/mL) for the original image (pink) and for the red, green and blue channels, respectively.

Initially, we studied the variation of test line intensity of AuNP based assays for three different PfHRP2 concentrations (10, 25 and 50 ng/mL) for the original image (pink) and its red, green, and blue channel (Fig. S1 A-F). The test line intensity variation with PfHRP2 concentrations while seen with naked eyes is shown in Figure S1A. As the pink colour is close to red, we neglect the red channel (Fig. S1B). We should pick either green (Fig. S1C) or blue channels (Fig. S1D). Since the ratio of test line intensity for the original image (pink) is proportional to the corresponding ratio of test line intensity for the green and blue channels (Fig. S1F), we can choose either a blue or green channel for analysis. We opted for the green channel as its

intensity is better than the blue channel (Fig. S1E). We repeated the same study for HRP and poly-HRP based immunoassays and opted for the blue channel (Fig. S2 A-F).



E)

PfHRP2 (ng/mL)	Test line intensity			
	Brown	Red	Green	Blue
$T_1 = 10$	43.016	35.349	41.36	52.833
$T_2 = 25$	47.796	39.761	47.744	56.502
$T_3 = 50$	74.494	61.779	78.007	87.838

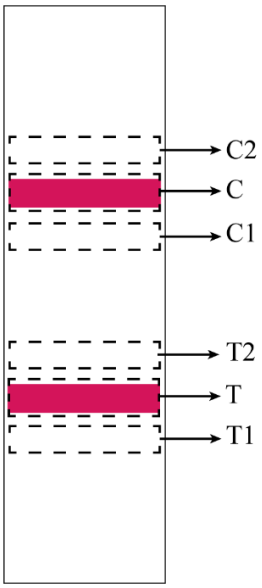
F)

PfHRP2	Ratio of test line intensity			
	Brown	Red	Green	Blue
—	—	—	—	—
T_1 / T_2	0.8999	0.8890	0.8662	0.9350
T_2 / T_3	0.6416	0.6436	0.6120	0.6432

Figure S2 Image analysis of test and control line intensities for HRP and PolyHRP based immunoassay using ImageJ (A-F)

A-D. Images showing the variation of test line intensity for three different PfHRP2 concentrations (5,25 and 100 ng/mL) for original image (pink), red, green and blue channel. E-F. The corresponding test line intensities and their ratio for three hCG concentrations (5,25 and 100 ng/mL) for the original image (pink), red, green, and blue channels.

Rectangular regions of interest (ROIs) of equal size were created on the test line and control line (to measure the actual signal) and on the immediate upstream and downstream of the test and control lines (to measure background signals), as illustrated in Figure S3. The signal was always reported as C – mean (C1 & C2).



Intensity	Test	Control
Measured	T	C
Background	TB = (T1+T2)/2	CB = (C1+C2)/2
Actual	T-TB	C-CB

Figure S3 **Schematic representation of the image analysis technique.**

B. Optimization of the concentrations of detection antibody conjugates for the various signal generation strategies

This section presents experimental results for strategies utilizing biotin-tagged detection antibodies, which bind to streptavidin-conjugated signal generation moieties.

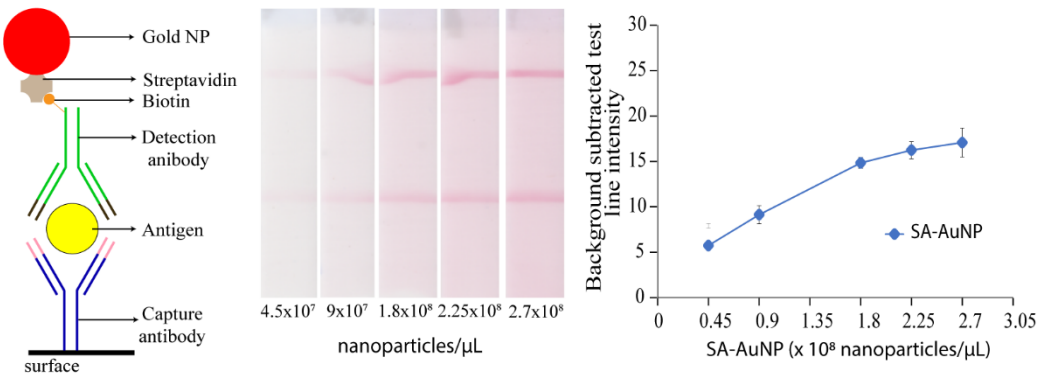


Figure S4 **The optimization of the concentration of SA-AuNP conjugate**
A. The immunoassay stack. B. Test strips showing the assay results for five different concentrations of detection antibody - AuNP conjugate. C. A plot of signal intensity against the concentration of the detection antibody - AuNP conjugate. The concentration of 1.8×10^8 nanoparticles/µL was selected as the optimum concentration as a higher concentration doesn't substantially improve the signal. For all the above experiments, error bars represent standard deviations (N = 3).

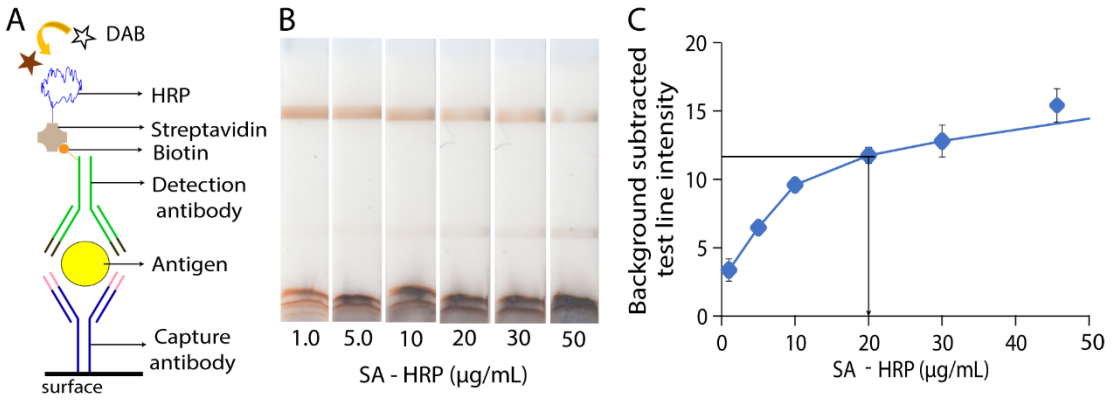


Figure S5 The optimization of the concentration of SA-HRP conjugate.
A. The immunoassay stack. B. Test strips showing the assay results for five different concentrations of detection antibody - HRP conjugate. C. A plot of signal intensity against the concentration of the detection antibody - HRP conjugate. The concentration of 20 µg/mL was selected as the optimum concentration as the higher concentration doesn't improve the signal substantially. For all the above experiments, error bars represent standard deviations (N = 3).

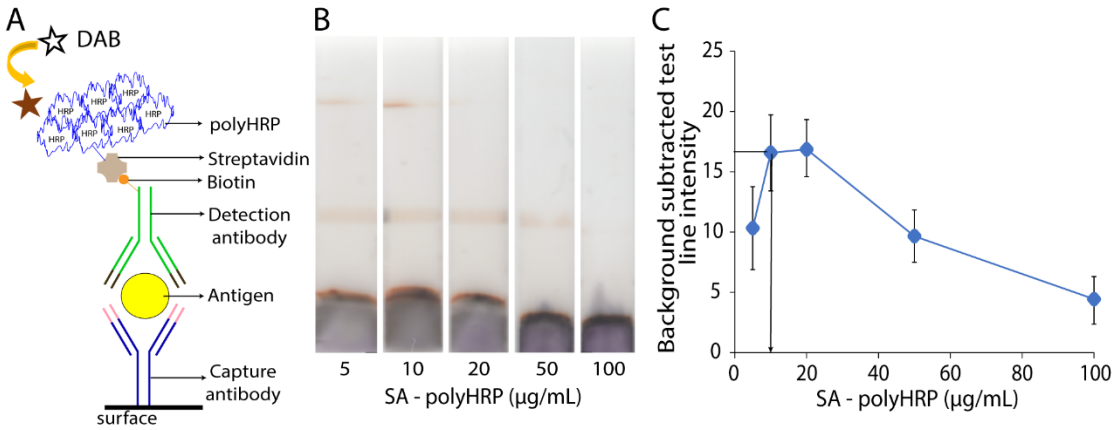


Figure S6 The optimization of the concentration of SA-polyHRP conjugate
A. The immunoassay stack. B. Test strips showing the assay results for five different concentrations of detection antibody - polyHRP conjugate. C. A plot of signal intensity against the concentration of the detection antibody - polyHRP conjugate. The concentration of 10 µg/mL was selected as the optimum concentration as the higher concentration doesn't improve the signal. For all the above experiments, error bars represent standard deviations (N = 3).

C. Design and assembly of the nitrocellulose strip comb

The nitrocellulose comb used to perform multiple lateral flow assays simultaneously was composed of three parts: i) an adhesive backing with laminate, ii) a set of 12 nitrocellulose strips, and iii) a wicking pad. The bottom part composed of an adhesive backing that was laser cut to remove laminate for sticking 12 nitrocellulose strips. The 12 nitrocellulose strips were then adhered on to this backing. The wicking pad having identical dimensions as that as the adhesive backing was then adhered on top of the backing. The nitrocellulose strips were thus sandwiched between the adhesive backing and the wicking pad. The distance between the nitrocellulose strips was so chosen such that the strips could be inserted into the 12 wells of a 96 well plate, and each All dimensions are provided in Figure S7.

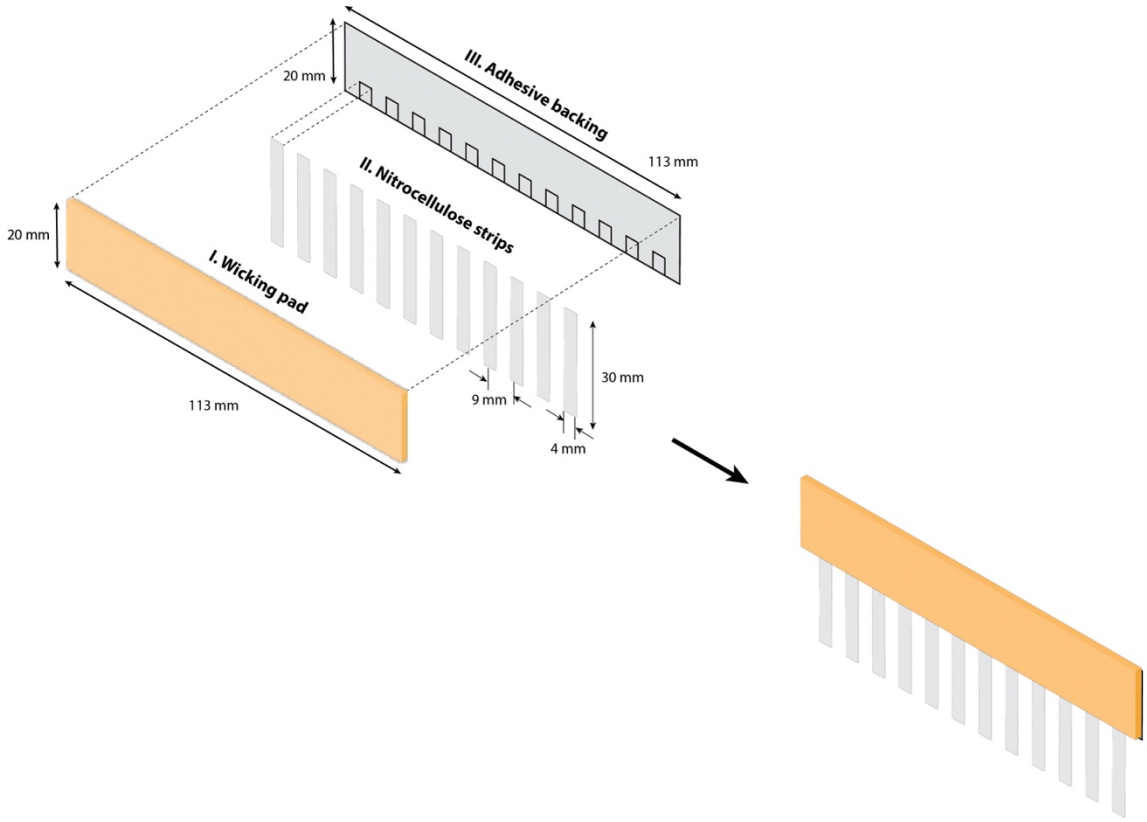


Figure S7. Design and dimensions of the nitrocellulose comb

D. Signal amplification strategies developed to detect PfHRP2 using anti-PfHRP2 detection antibodies directly conjugated to the gold or enzyme labels.

We compared the standard lateral flow gold-nanoparticle based immunoassay with gold enhancement technique and HRP enzyme-based immunoassay, as illustrated in Figure S5.

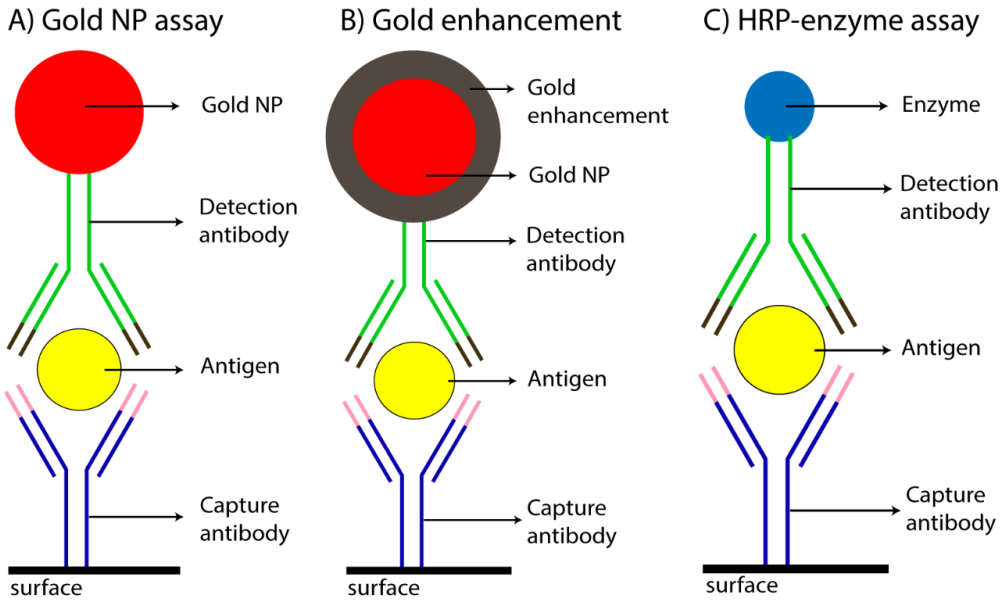


Figure S8 Comparison of various immunoassay strategies.

A. Gold nanoparticle immunoassay. **B.** Gold enhancement of gold nanoparticles. **C.** HRP-based signal enhancement strategy.

D1. Direct Gold nanoparticles - Assay protocol, results and discussion

A sandwich immunoassay was developed to detect PfHRP2 antigen using the anti-PfHRP2 antibody and gold nanoparticle conjugate (IgG-AuNP). A comb of nitrocellulose strips attached to a cellulose wicking pad with the help of a backing card was manually dipped into the first row (A) of a 96-well plate, as shown in Figure S6 (A). The first row (A) of the well plate consists of 30 µL of blank (Column 1) and 30 µL of PfHRP2 antigen of increasing concentration ranging from 0.5 to 5000 ng/mL (from Column 2 to 12); each of these antigen solutions was pre-equilibrated with the 10 µL of $1.8x10^8$ nanoparticles/µL of IgG-AuNP conjugate. The concentration of anti-PfHRP2 IgG gold conjugate for the standard PfHRP2 lateral flow immunoassay was optimized and found to be $1.8x10^8$ nanoparticles/µL. The imbibition of the analyte-antibody conjugate resulted in the generation of test and control line indicating the presence of antigen and the generation of only the control line indicating the absence of antigen after dipping in row (A) for 10 minutes as shown in Figure S6 (B).

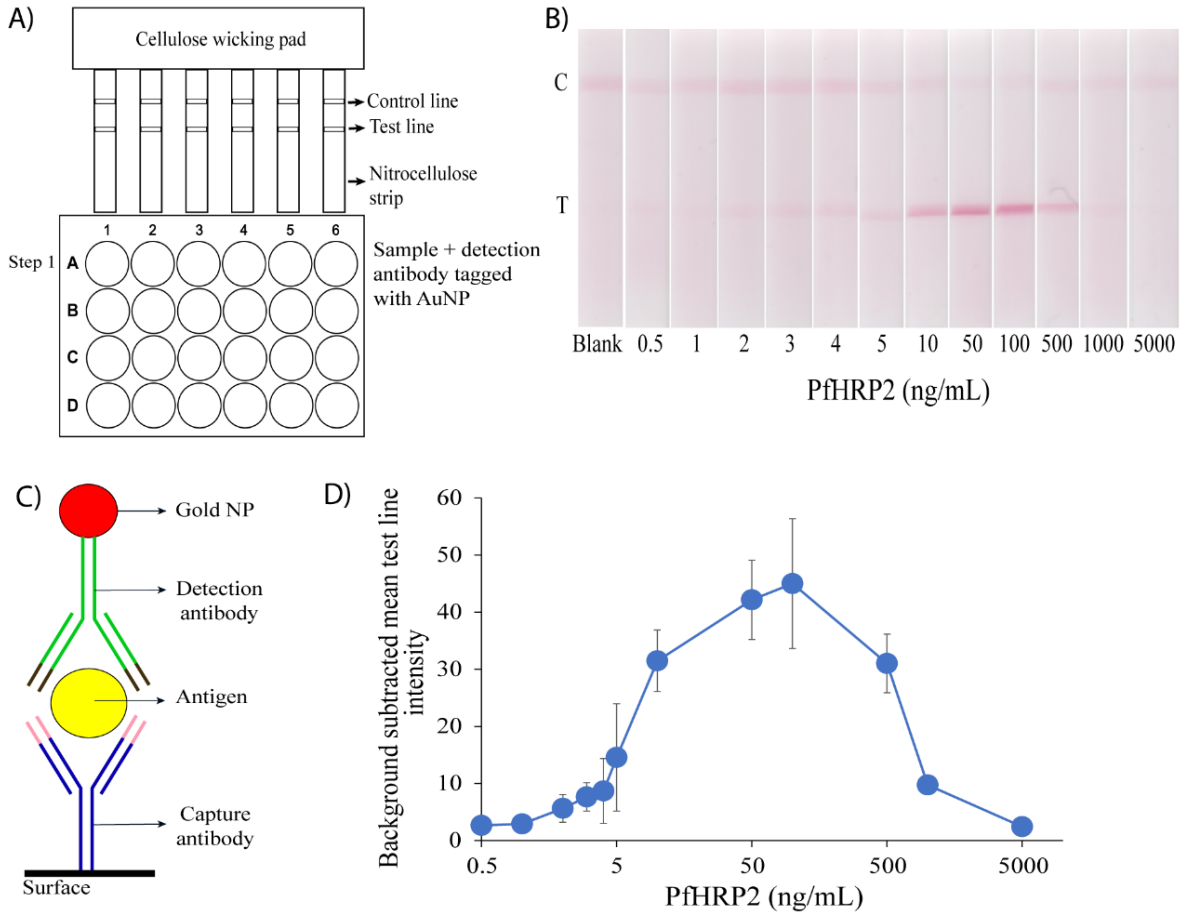


Figure S9 **Standard lateral flow immunoassay using 40 nm Innova coat gold nanoparticles.**

A. The assay protocols. **B.** The image of the test strips after the assay for the increasing concentration of PfHRP2 antigen. **C.** the PfHRP2 immunoassay stack and **D.** the corresponding calibration curve.

The corresponding test line immunoassay stack consisting of a primary capture antibody, PfHRP2 antigen, and a detection antibody conjugated with gold nanoparticle was shown in Figure S6 (C). A calibration curve obtained by plotting PfHRP2 antigen concentration against the background-subtracted mean test line intensity for IgG-AuNP based assay was shown in Figure S6 (D). Notably, the assay shows the presence of a 'hook effect' after PfHRP2 concentration of 100 ng/mL. The nitrocellulose comb from the above IgG-AuNP based assay was washed twice with PBST by manually dipping it in the subsequent rows C and D for 4 minutes each (Fig. S7 (A)). This washing step was crucial for removing any unbound IgG-AuNP conjugate as this non-specific binding interferes with the further amplification.

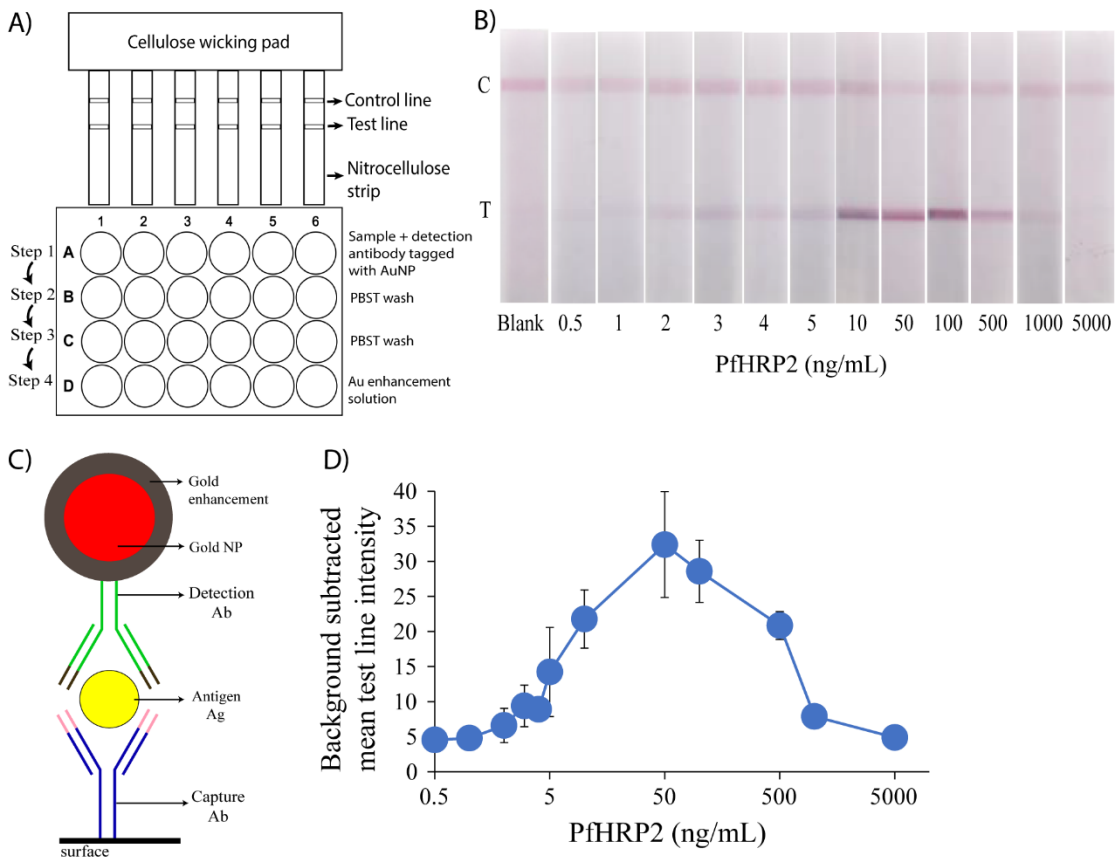


Figure S10 Gold enhancement strategy

A. The assay protocols. **B.** The image of the test strips after the gold enhancement for the increasing concentration of PfHRP2 antigen. **C.** the PfHRP2 immunoassay stack with signal enhancement and **D.** the corresponding calibration curve.

Finally, the comb was moved to row D containing the gold enhancement reagent. When the gold enhancement solution flows through the strips, the reduction of Au ions (present in the enhancement solution) occurs at the test and control lines. This leads to the formation of the large size nanocrystals at or near the site of AuNPs (which acts as development nuclei), resulting in an enhanced signal. The enhanced signal is shown in Figure S7 (B), and the corresponding test line immunoassay stack is shown in Figure S7 (C). A plot of PfHRP2 antigen concentration against the background-subtracted mean test line intensity for gold enhancement assay was shown in Figure S7 (D).

D2. Horseradish peroxidase (HRP) enzyme-based enhancement - Assay protocol, results and discussion

A sandwich immunoassay to detect PfHRP2 antigen using horseradish peroxidase-conjugated anti-PfHRP2 IgG antibody (IgG-HRP) was developed. A comb of nitrocellulose strips attached to a cellulose wicking pad with the help of a backing card was manually dipped sequentially in the rows of a 96-well plate, as shown in Figure S8 (A). The first row (A) of the well plate consists of 30 µL of blank (Column 1) and 30 µL of PfHRP2 antigen of increasing

concentration (from Column 2 to 12); each of these solutions was pre-equilibrated with the 10 µL of 10 µg/mL of IgG-HRP conjugate. The imbibition of the analyte-antibody conjugate resulted in forming a test line immunoassay stack consisting of a primary capture antibody, PfHRP2 antigen, and IgG-HRP detection antibody conjugate, as shown in Figure S8 (C). This was followed by PBST washing by moving the comb to rows B and C (at an interval of 4 minutes each) for removing non-specific binding. Finally, the comb was moved to row D containing the CN/DAB substrate. This resulted in the generation of test and control line indicating the presence of antigen and generation of only the control line indicating the absence of antigen, as shown in Figure S8 (B). A plot of PfHRP2 antigen concentration against the background-subtracted mean test line intensity for IgG-AuNP based assay was shown in Figure S8 (D).

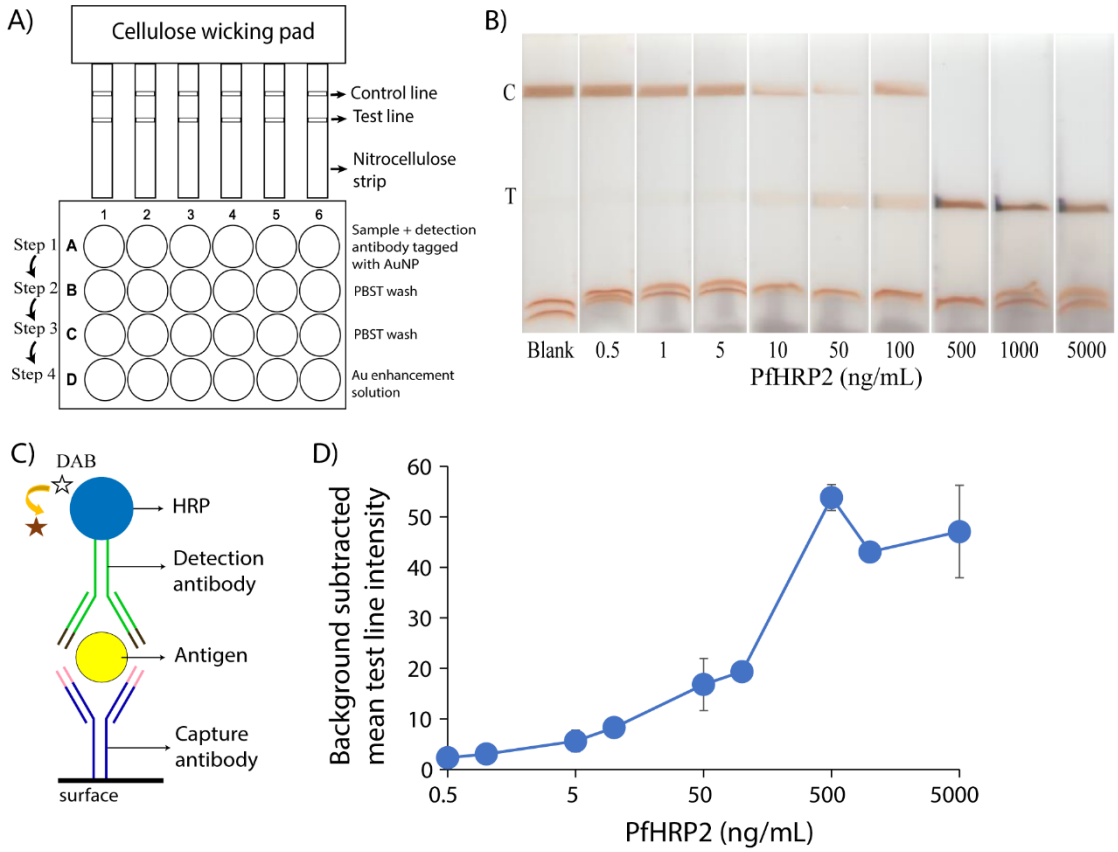


Figure S11 HRP enzyme-based assay where the detection antibody was directly conjugated to HRP enzyme.

A. The assay protocols. **B.** The image of the test strips after the gold enhancement for the increasing concentration of PfHRP2 antigen. **C.** the PfHRP2 immunoassay stack with signal enhancement and **D.** the corresponding calibration curve.

The LOD of the above signal enhancement strategies was determined and compared using the method introduced by Holstein et al.[143]. LOD of standard lateral flow AuNP assay, Au-based enhancement of AuNP, and HRP based enhancement was shown in Table S1, and the corresponding graphs for determining the LOD are shown in Figure S9 (A-D).

Table S1 Comparison of the LOD for each signal enhancement strategy

S.No	Signal enhancement strategy	LOD (Holstein's) ng/mL	LOD 95 % confidence interval
1	Gold nanoparticles	4.16	[2.93 ng/mL, 5.68 ng/mL]
2	Gold enhancement	3.08	[1.82 ng/mL, 4.76 ng/mL]
3	HRP enzyme-based	27.57	[14.69 ng/mL, 50.39 ng/mL]

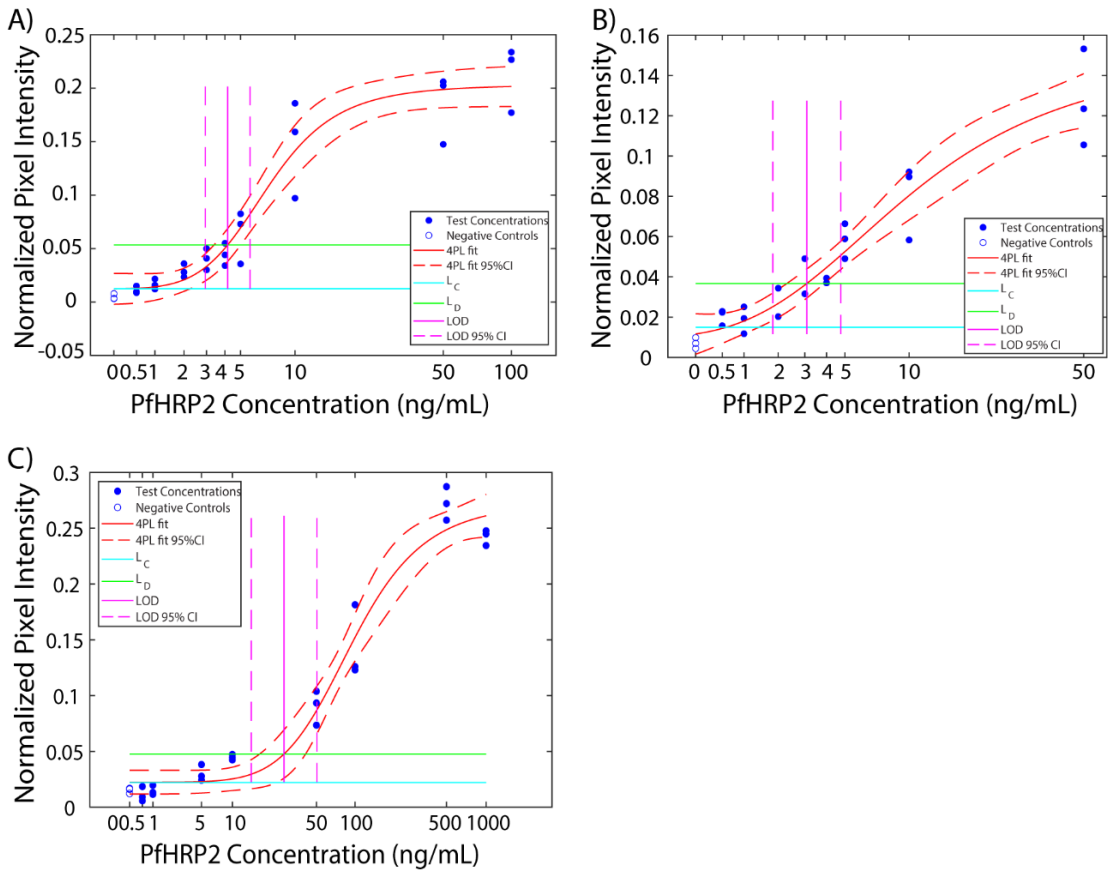


Figure S12 Limit of detection for various strategies (A-D).

A. *LOD for standard AuNP assay.* ***B.*** *Au enhancement of SA-AuNP, SA-HRP and SA-polyHRP based enhancement, respectively.*

These results show that the Au-based enhancement of AuNP gives a better LOD than other enhancement techniques. From the tables (table 1 and S9), it is interesting to note that the assays with a direct conjugation scheme have a better LOD than the biotinylated assays. Nevertheless, the latter was performed for an effective comparison of the signal enhancement strategies. We started our work with the direct HRP conjugated assay, but its LOD was poor than the standard gold nanoparticle assay. To improve the LOD, we developed polyHRP based

assay. To compare the direct HRP enzyme assay with the polyHRP assay, for a given volume (10 μL) of detection antibody conjugate, the amount of detection antibody (IgG) used in both the assays should ideally remain the same. To accomplish that, we calculated the concentration of biotinylated antibodies that should be supplied and found out to be 8 μg/mL. Hence, we decided to use this concentration (8 μg/mL) of biotinylated detection antibody throughout our comparison study.

E. Cost of Labels

Table S2. Comparison of Costs of Labels

S. No.	Signal enhancement strategy	Cost of the labels per 10 strips (USD)**
1.	SA-AuNP (Base case)	12
2.	Gold enhancement of SA-AuNP	14
3.	SA-HRP enzyme	0.3
4.	SA-polyHRP enzyme	1.6

*** Costs in INR for reagents purchased in India were considered and converted to USD assuming a conversion rate of INR 83 = 1 USD*

The retail costs incurred in the purchase of the various labels in Table S2. Nanoparticle-based systems are more expensive compared to the HRP-DAB systems; the cost of HRP-DAB and polyHRP-DAB labels are approximately 2 and 1 orders of magnitude less than nanoparticle-based labels.

F. Enzyme-kinetics study for complete conversion of DAB using HRP enzyme.

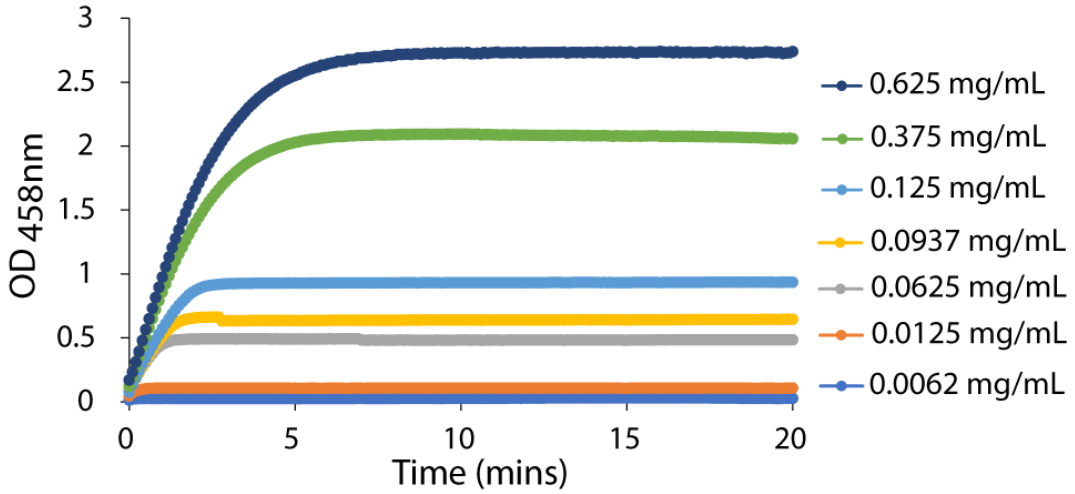


Figure S13. ***Enzyme-kinetics study for complete conversion of DAB using HRP enzyme.***

Enzyme-kinetics study for various HRP enzyme concentrations using time-lapse imaging.

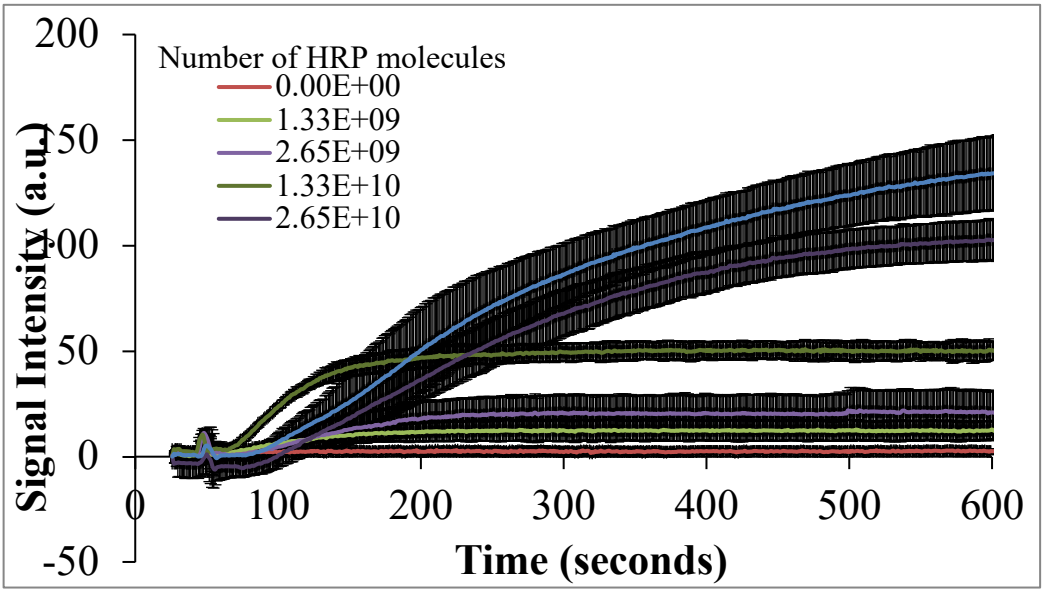


Figure S14. Kinetics study for the development of signal for various concentrations of HRP enzyme directly spotted on the nitrocellulose membrane. Error bars corresponds to n<3.