Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

A Paper Biosensor for Visual Detection of Glucose-6-Phosphate Dehydrogenase from Whole Blood

Dawn White, Mehdi Keramane, Alfredo Capretta* and John D. Brennan*

Biointerfaces Institute, McMaster University, 1280 Main St W, Hamilton, ON, L8S 4L8, Canada

Section S1: G6PD concentrations used in this study.

Whole sheep blood samples were prepared according to WHO G6PD classification levels shown below.

Table S-1. G6PD concentrations (U/dL) used in this study.

Units G6PD/dL Blood	Units G6PD/g hemoglobin*	WHO category[1]	Classification[1,2]
0	0.00	NA	Control
4	0.26	I, II	Deficient (<10%)
60	3.87	III	Intermediate (10-60%)
132	8.55	IV	Normal (60-100%)
220	14.49	V	Above (>150%)

* based on the average of the range of normal Hb levels in men and women (~15 g Hb/dL blood)

We based this proof-of-concept assay on an average hemoglobin level. Variation in hemoglobin levels between different geographic populations would require optimization based on the population being considered.[2,3]

Section S2: Colorimetric dye selection, assay reagent mixture optimization and data evaluation.

1-methoxy-5-methylphenazinium methyl sulfate (1-PMS) was chosen over PMS as the electron carrier since PMS solutions require storage at -20 °C and protection from light while 1-PMS solutions can be stored at room temperature without protection from light for ≥ 3 months.[5]

The choice of tetrazolium salt used in the final paper biosensor was based on the intensity of the resulting colored formazan dye when mixed with the blood sample. The popular tetrazolium salts for dehydrogenase assays, namely 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), iodonitrotetrazolium chloride (INT), 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) and 2,2'-Dibenzothiazolyl-5,5'-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)ditetrazolium disodium salt (WST-5), were first tested in solution using human serum (Innovative Research); all worked well but only MTT and XTT produced a color that was easily discernable from background, purple and yellow, respectively (Figure S-1A). Of MTT and XTT, only MTT produced a darker color above the natural color of the whole blood sample, providing the best visual output (Figure S-1B); the yellow color of XTT mixed with the red blood sample provided no contrast.

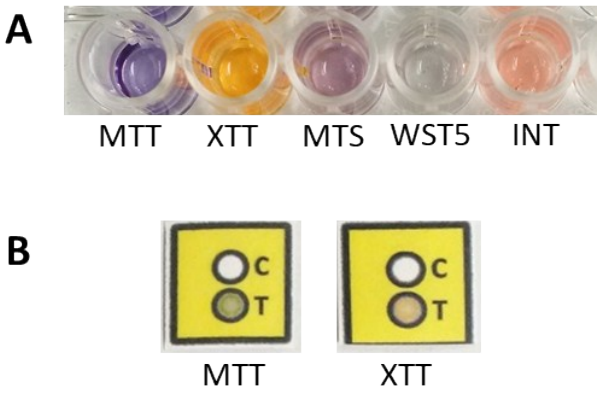


Figure S-1. Tetrazolium salt testing. **(A)** Color output of various tetrazolium salts at above normal G6PD concentrations in human serum. **(B)** Choosing MTT over XTT as the tetrazolium salt for the paper-based assay during preliminary (not yet optimized) testing of above normal G6PD samples.

The concentration of each component of the assay reagent mixture was initially based on work by Zhu *et al*.[6] In preliminary testing using human serum in solution (as used in the tetrazolium salt selection assays), this method worked well. Sensitivity was lower when the assay was moved to paper with whole sheep blood samples. The original reagent concentrations were therefore modified to regain a color difference between the G6PD designations (deficient, intermediate, normal and above normal) on paper. The reagent combination called "Mix #6" (Table S-2) was chosen for further investigations as this mixture provided the clearest visual distinction between G6PD designations (Figure S-2). In this experiment, freshly prepared reagents mixes were applied to the paper biosensor, dried for 4 hours and immediately used for testing whole sheep blood samples that were diluted directly in 50 mM Tris (pH 7.5).

Table S-2. G6PD assay reagent mixture concentration optimization.

Reagent*	Mix #1	Mix #2	Mix #3	Mix #4	Mix #5	Mix #6	Mix #7
1-PMS	0.1	0.1	1	0.1	0.1	0.1	0.1
MTT	6.1	12.2	12.2	6.1	6.1	6.1[t]	12.2
$MgCl_2$	10	10	10	25	10	10	10
NADP	0.25	0.25	0.25	0.25	0.1	0.25	0.25
G6P	1	1	1	1	1	2	2
Tris pH 7.5	50	50	50	50	50	50	50

*All reagent concentrations in mM.

[t] MTT was removed from the reagent mixture when dried on paper and was added as a separate component at 2x the stated concentration as part of the blood buffer solution (below).

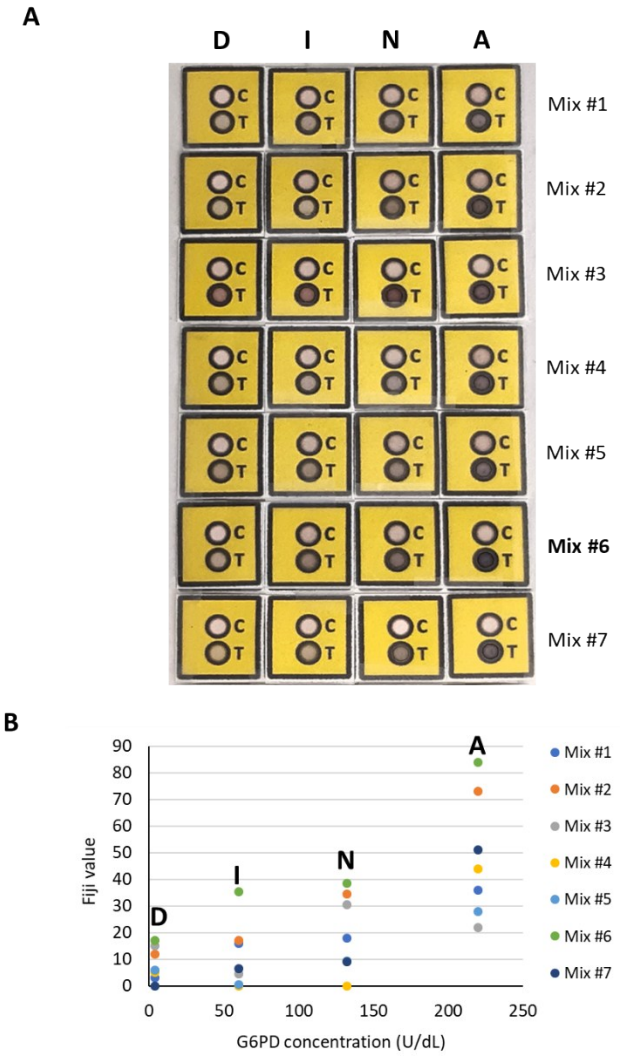


Figure S-2. G6PD assay reagent mixture testing. **(A)** The best visual distinction between G6PD designations was achieved with Mix #6 (bold). **(B)** Color intensity of test spots for the different assay mixtures for each G6PD designation as measured using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal).

During initial paper-based testing described above, MTT was included in the assay mixture, dried on the test spot of the paper sensor and used immediately. When the test spots were prepared for longer-term use, it was observed that MTT was not stable in the assay mixture (specifically reacting with the Tris component), spontaneously converting to its formazan dye within 7 days (Figure S-3 top; and for comparison, Figure S-3 bottom, MTT prepared in water).

Following this finding, 25 mg of MTT solid was pre-weighed into a reagent tube to which 5 mL of 50 mM Tris buffer, pH 7.5 (blood diluent buffer) was added to achieve two times the final MTT concentration noted in Mix #6. The remaining assay reagents (Mix #6 without MTT: 0.1 mM 1-PMS, 10 mM $MgCl_2$, 0.25 mM NADP, 2 mM G6P, 50 mM Tris, pH 7.5 and 5% (w/v) pullulan) were prepared, spotted and dried as described in the test spot for all further assays.

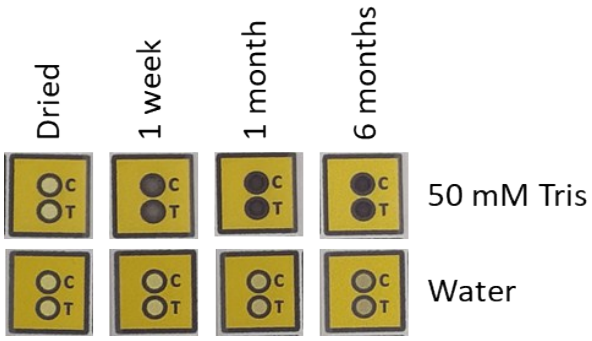


Figure S-3. MTT stability testing. Stability of MTT solutions dissolved in 50 mM Tris (**top**) or water (**bottom**) after drying on paper. Both the "C" and "T" spots were used for testing the dried MTT solutions in duplicate.

In the initial exploratory experiments (like in Figure S-2 and the following figures), G6PD detection assay results were taken at the end-point (when samples had just dried) in ambient room lighting using automatic settings on a Samsung Galaxy A8 smartphone. Fiji[7] was used to quantify the intensity of the sample spots after inverting the image. Since the color of the sample was not uniform across the spot, the "color average" function was used first to establish a single hue across the region of interest (the test sample spot). A measurement was then taken for color intensity. The background intensity (0 U/dL) was subtracted from the value for all other G6PD concentrations on the same test strip prior to graphical representation.

Section S3: Assay optimization.

A. Whole blood diluted directly in buffer.

Dilution of the whole blood sample played a major role in the sensitivity of the assay. Lesser dilutions (1:2–1:10, blood:buffer) show fast and robust enzyme activity with little distinction

between G6PD designations (not shown); greater dilutions (1:50-1:100, Figure S-4) allowed better distinction, with the 1:100 dilution giving the best graded response visually. Here, whole blood was diluted directly in 50 mM Tris (pH 7.5) containing freshly dissolved MTT (MTT buffer solution) and then applied to reagent Mix #6 (without MTT) previously dried in the test spot.

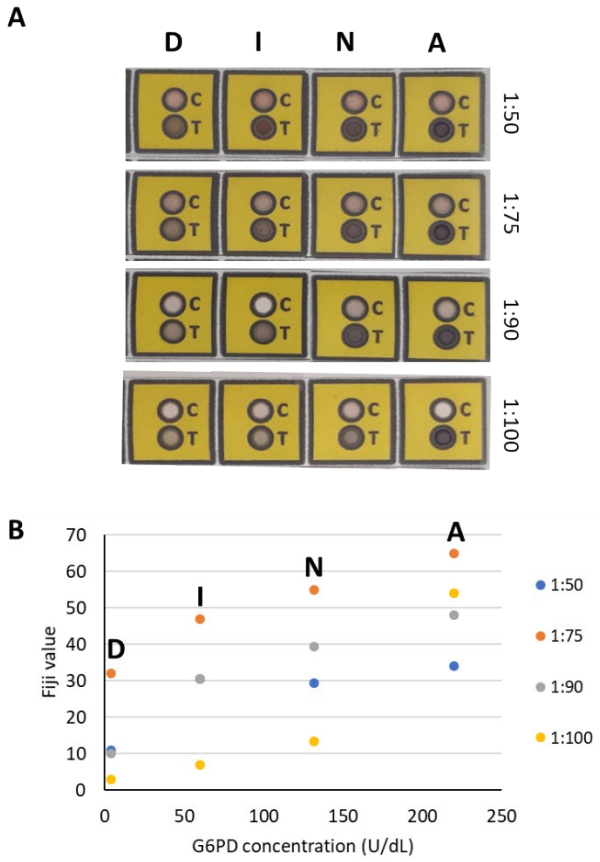


Figure S-4. Effect of whole blood dilution on distinguishing G6PD levels. **(A)** Color development of a standard assay with blood dilution indicated on the right. **(B)** Color intensity of test spots for each G6PD designation of a typical assay as measured using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal). Note that the data point for 1:50 and 1:90 at 60 U/dL G6PD overlap on the graph.

B. Whole blood eluted from Whatman 903™ Protein Saver card into buffer.

The dilution effect was also tested using the final assay format involving the application of the G6PD-spiked blood samples to Whatman 903™ Protein Saver card. Here, 75 µl of blood was

applied to individual circles of the Whatman 903™ Protein Saver card (as this volume filled the sample application area) and added to varying volumes of MTT buffer solution (Figure S-5). The best color distinction, most closely matching the color generated when whole blood was diluted directly in MTT buffer solution at 1:100 (Figure S-4 above), was obtained when 5 ml of MTT buffer solution was used. Note that an exact blood:buffer dilution cannot be reported since the amount of blood eluted from the card into the buffer cannot be known.

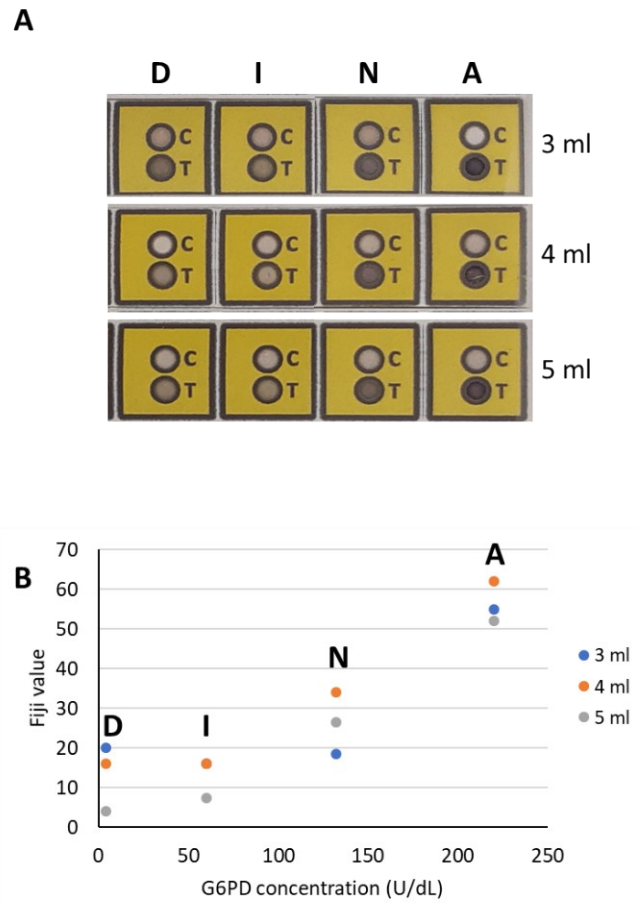


Figure S-5. Maintaining best sample dilution with whole blood applied to paper. **(A)** Color development of a standard assay with MTT buffer solution volume indicated on the right. **(B)** Color intensity of test spots for each G6PD designation of a typical assay as measured using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal). Note that the 3 ml and 5 ml data points for intermediate G6PD levels overlap on the graph.

C. Decreasing whole blood volume applied to Whatman 903™ Protein Saver card.

A test was conducted to determine the effect on detection assay sensitivity when lower blood volumes were applied to the Whatman 903™ Protein Saver card while keeping the 5 ml volume of MTT buffer solution constant. A blood volume of 60 µl also filled the sample circle and generated a similar response to that when 75 µl of blood was used (as in the developed assay, Figure S-6A), showing a darkening color gradient as G6PD concentration increased. Decreased blood volumes (50 µl and 25 µl) generated a simple yes/no answer, where only samples that were normal or above normal produced a color change (Figure S-6B and C).

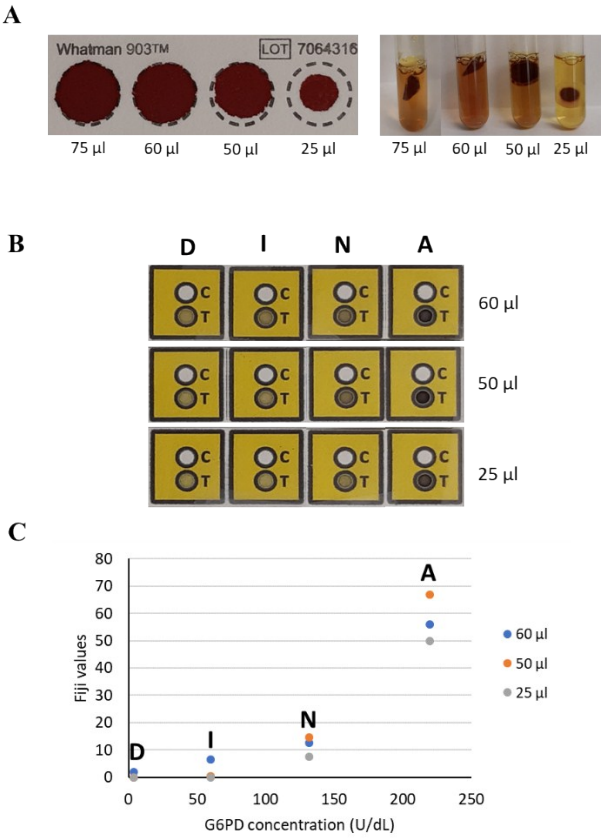


Figure S-6. Decreasing blood volume used in the detection assay. **(A) (Left)** Visual of how the different blood volumes fill the circles of the Whatman 903™ Protein Saver card. **(Right)** Corresponding samples in 5 ml of MTT buffer solution. **(B)** Color development of a standard assay with applied whole blood volume indicated on the right. **(C)** Color intensity of test spots for each G6PD designation of a typical assay as measured using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal).

D. Scaling down the assay.

In the final iteration of the assay presented in the main paper, the volumes of MTT buffer solution and whole blood were scaled down to 1 ml and 10-15 µl, respectively, without loss of performance. This would reduce reagent cost and required blood sample.

E. Elution time

Using the final configuration of the assay, elution time of the blood sample from the Whatman 903™ Protein Saver card into the MTT buffer solution was investigated. Prepared intermediate, normal and above normal G6PD samples were eluted for 5-20 minutes before evaluation. The signal generated within each G6PD designation remained relatively constant (Figure S-7). Elution times greater than 20 minutes were not tested since the aim was to create a POC device that required minimal assay time.

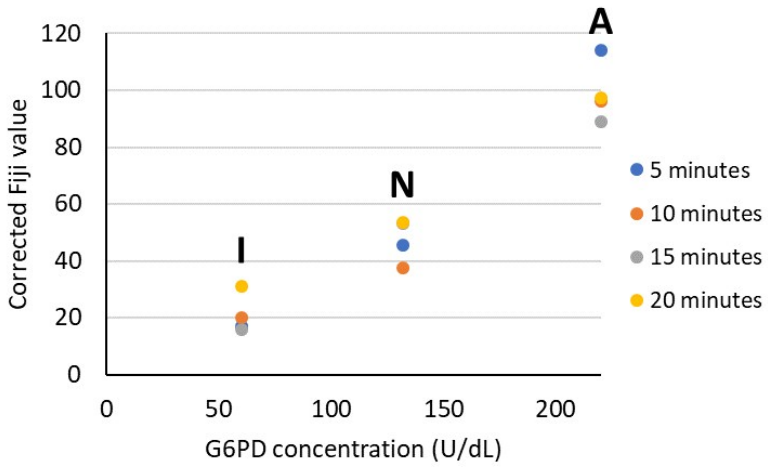


Figure S-7. Sample elution time. Color intensity of test spots for each G6PD designation of a typical assay as measured using Fiji (I = Intermediate; N = Normal; A = Above normal).

Section S4: Paper sensor stability.

To evaluate sensor stability, the assay reagent mixture (Mix #6 without MTT) was applied to enough test spots to provide for 6 weeks of G6PD assay testing. The papers were kept at ambient

temperature and humidity in the dark until use. G6PD detection was performed weekly at ambient temperature and humidity (Figure S-8).

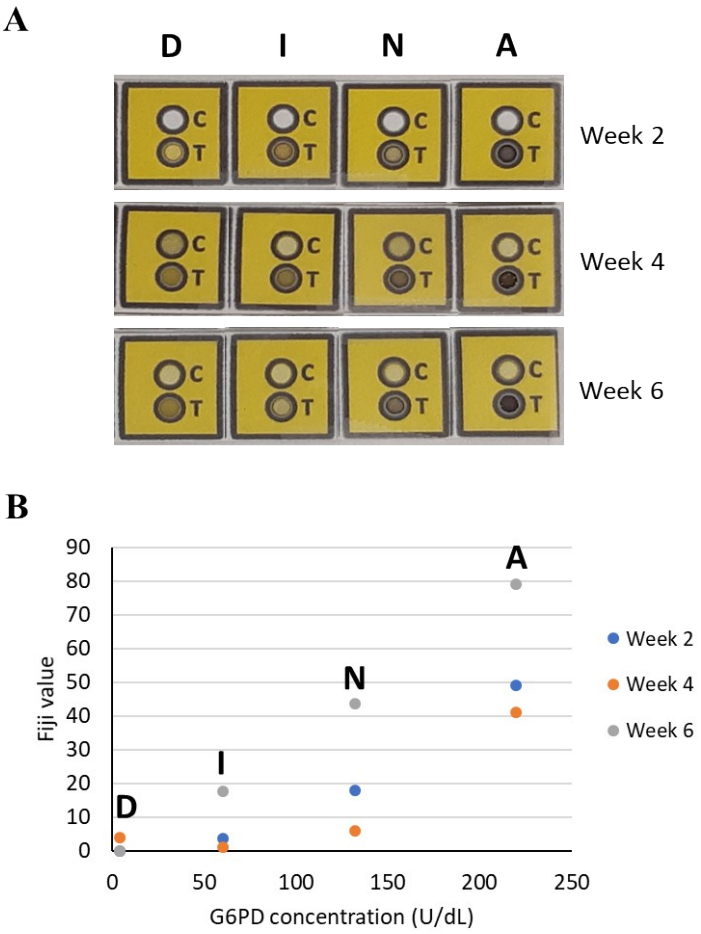


Figure S-8. G6PD detection over six weeks using assay reagents dried on paper. **(A)** Color development of the standard assay over the course of 6 weeks. (**B)**. Color intensity of test spots for each G6PD designation as measured using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal). Note that the value for Deficient samples at weeks 2 and 6 overlap on the graph.

Section S5: Pentose phosphate pathway dehydrogenase specificity.

G6PD is the first and rate-controlling enzyme in the pentose phosphate pathway[8]. A second dehydrogenase in the pathway, 6-phosphogluconate dehydrogenase (6PDG), can be effectively inhibited (K_i of 0.5 µM[9]) using 6-aminonicotinamide (6-AN; Millipore Sigma) to show that the enzyme activity seen in this assay was due to G6PD. Using a solution-based assay with 132 U/dL

G6PD (pure enzyme) in PBS, we observed that addition of 6-AN (concentration range of 5 µM to 1 mM) had no effect on G6PD activity (Figure S-9, 1-6). In the presence of plain whole sheep blood (which is effectively G6PD deficient), with or without 100 µM inhibitor, there was no color change to indicate 6PDG activity (Figure S-9, 10-12). From these results, we feel confident that the color change we see in our assay is due to G6PD alone.

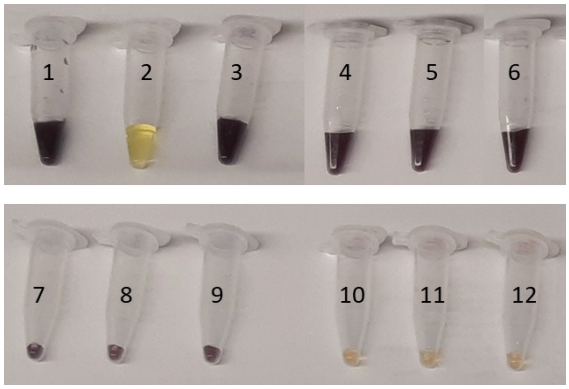
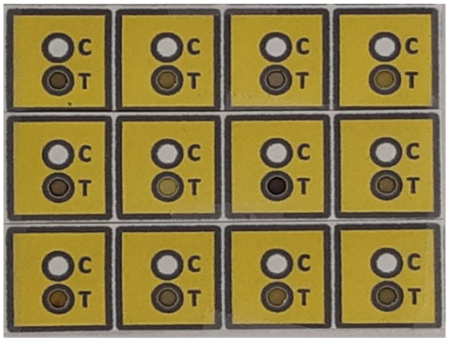


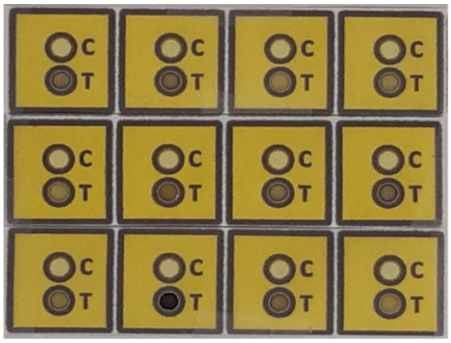
Figure S-9. G6PD activity in the presence of 6-aminonicotinamide (6-AN). (Top) Solution assay using pure G6PD (132 U/dL) in the presence of increasing inhibitor concentration: 1. Positive control (G6PD, no 6-AN); 2. Negative control (no G6PD, with 5 µM 6-AN); 3. Inhibited reaction (G6PD, 5 µM 6-AN); 4. Inhibited reaction (G6PD, 25 µM 6-AN); 5. Inhibited reaction (G6PD, 100 µM 6-AN; 6. Inhibited reaction (G6PD, 1 mM 6-AN). (Bottom) Solution assay using whole sheep blood samples spiked with 220 U/dL G6PD (left three samples) or un-spiked (right three samples): 7. Positive control; 8. "Negative" control (color change still occurred since the blood was spiked with G6PD, 100 µM 6-AN had no effect); 9. Inhibited reaction (100 µM 6-AN); 10. Positive control (no color change since sheep blood tests as G6PD deficient; any 6PDG naturally found in the blood sample did not induce a color change); 11. Negative control (100 µM 6-AN); 12. Inhibited reaction (100 µM 6-AN).

Section S6: G6PD designation blind testing by trained and untrained users.

Twenty-four random blind G6PD samples were assessed by a trained user without any visual clues (no color card, no completed G6PD detection assay). Predicted versus actual G6PD designations were compared (Figure S-10): 19/23 samples were correctly assessed.



Predicted: Normal Actual: Normal	Predicted: Intermediate Actual: Intermediate	Predicted: Normal Actual: Normal	Predicted: Deficient Actual: Deficient
Predicted: Normal Actual: Normal	Predicted: Deficient Actual: Deficient	Predicted: Above Actual: Above	**Predicted: Normal** **Actual: Intermediate**
Sample ruined Not assessed	Predicted: Normal Actual: Normal	Predicted: Intermediate Actual: Intermediate	**Predicted: Normal** **Actual: Intermediate**



Predicted: Normal Actual: Normal	Predicted: Normal Actual: Normal	Predicted: Deficient Actual: Deficient	Predicted: Intermediate Actual: Intermediate
Predicted: Deficient Actual: Deficient	**Predicted: Normal** **Actual: Intermediate**	**Predicted: Intermediate** **Actual: Normal**	Predicted: Normal Actual: Normal
Predicted: Intermediate Actual: Intermediate	Predicted: Above Actual: Above	Predicted: Intermediate Actual: Intermediate	Predicted: Deficient Actual: Deficient

Figure S-10. Blind testing by a trained user. Two, twelve sample grids were prepared on different days and assessed. One sample was ruined and not included.

Using a color card only (Users #1-4) or a color card and a completed G6PD detection assay, (Users #5-7) untrained users predicted the G6PD designation from 20 random samples. The most discrepancies occurred when distinguishing between intermediate and normal samples (Tables S-3 and S-4).

Table S-3. Untrained user G6PD designation assessment.

	Deficient	Intermediate	Normal	Above	Total
Actual	**4**	**7**	**6**	**3**	**20**
User #1	4	5	3	3	15
User #2	2	4	2	3	11
User #3	4	5	3	3	15
User #4	4	5	5	1	15
User #5	4	5	4	3	16
User #6	3	6	5	3	17
User #7	3	6	5	3	17

Table S-4. Untrained user specific misidentification.

	Deficient/Intermediate	Intermediate/Normal	Normal/Above
User #1	0	2	3
User #2	2	4	3
User #3	0	3	2
User #4*	1	2	0
User #5	1	2	1
User #6	1	2	0
User #7	1	2	0

*This untrained user uniquely misidentified two Intermediate/Above.

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