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A paper-based biosensor for visual detection of glucose-6-phosphate dehydrogenase from whole blood†

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Screening for a deficiency of glucose-6-phosphate dehydrogenase (G6PD) in red blood cells is vital for determining the potentially life-threatening presence of congenital, hereditary or induced hemolytic anemias. In this study, a "sample-to-readout" paper-based point-of-care (POC) colorimetric biosensor was developed for direct detection of G6PD in whole blood by simple visual comparison to a color card. The G6PD paper sensor was highly stable with no observable loss in performance after room temperature storage for at least 6 weeks, and worked equally well at room temperature and 37 °C. The simple printed paper format and the stability of the colorimetric reagents facilitates scalable manufacturing. The ability to utilize well established sample collection and preparation protocols along with a colorimetric visual readout should facilitate future transfer of this proof-of-concept POC biosensor to remote or resource-poor locations.

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most prevalent enzymopathy in humans.[1] Greater than 400 million people globally, mostly in sub-Saharan Africa, tropical Asia and the Middle East, are affected by this genetic condition, many of whom are undiagnosed.[2] Undetected, this enzyme deficiency can escalate and contribute to many life-threatening conditions, including neonatal jaundice (potentially leading to brain damage), hemolytic anemia, drug-induced hemolysis, and infection-induced hemolysis associated with viruses (*e.g.* hepatitis, causing liver inflammation), microbes (*e.g. Bartonella bacilliformis*, causing Carrión disease) and protozoal parasites (*e.g. Plasmodium* species, causing malarial infections).[3]

G6PD deficiency tests are classified as quantitative, semi-quantitative or qualitative. Quantitative testing, the definitive gold standard that can discriminate between normal (60–100%), intermediate (10–60%) and deficient (<10%) G6PD enzyme activity levels, as defined by the World Health Organization (WHO),[4] can only be performed in a specially equipped laboratory, where patient blood samples are processed and analyzed using a UV or visible spectrophotometric assay (*e.g.*, Trinity Biotech 345 and 400, respectively). The most widely-used screening test, even in the field, is the fluorescent spot test (Trinity Biotech 203 among others), a semi-quantitative test in use since its introduction in the 1960s.[5] However, this test is not simple or inexpensive, as it requires refrigeration of reagents, a water bath to maintain a constant temperature, a UV lamp and extensive user training.[6]

At present, there are no commercially available rapid point-of-care (POC) tests that can be used reliably in the field in resource limited locations. Qualitative tests currently on the market include the Alere BinaxNOW® G6PD test and the Access Bio CareStart™ G6PD deficiency screening test. Both tests have been trialled extensively[7,8] however, while the simple "yes/no" result of these tests makes them user-friendly, they are limited to identifying severe deficiencies. The BinaxNOW® kit requires a cold chain, pipettes and has a narrow operating temperature, making its use in environmentally variable settings unreliable.[9] The CareStart™ test performs well in the field[9,10] but has been reported to be difficult to interpret and in need of a control line.[6,11,12] What is urgently needed is a reliable POC test that can provide a semi-quantitative visual readout and is amenable to use by untrained users in the field.

One of the most commonly used assay formats for monitoring dehydrogenase enzyme activity involves colorimetric detection of a formazan dye product using a suitable substrate for the dehydrogenase and either β-nicotinamide adenine dinucleotide (NAD) or β-nicotinamide adenine dinucleotide phosphate (NADP) as a coenzyme.[13,14] For G6PD, colorimetric detection of enzyme activity can be performed as outlined in

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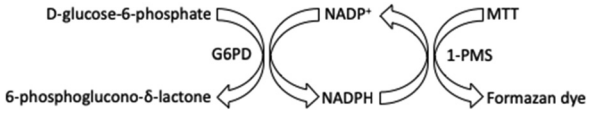

Fig. 1 Colorimetric assay for detection of G6PD.

Fig. 1. G6PD catalyzes the conversion of D-glucose-6-phosphate (G6P) to 6-phosphoglucono-δ-lactone with the simultaneous formation of NADPH (reduced form) from $NADP^+$. In the presence of 1-methoxy-5-methylphenazinium methylsulfate (1-PMS), the generated NADPH converts a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) to a brightly colored formazan dye.

In this work, we provide proof-of-concept for a POC test that allows visual detection of G6PD deficiency, that is amenable to direct testing of whole blood and does not require any sophisticated instrumentation. The POC test requires simple addition of a fresh blood sample into a single solution followed by direct application of the diluted blood solution to a paper-based sensor, allowing for a semi-quantitative colorimetric readout within 5–40 minutes.

Experimental

Materials

β-Nicotinamide adenine dinucleotide phosphate (NADP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Whatman #1 filter paper and Whatman 903™ Protein saver cards were purchased from Millipore-Sigma. 1-Methoxy-5-methylphenazinium methylsulfate (1-PMS) was purchased from Dojindo Molecular Technologies. D-Glucose-6-phosphate (G6P) was purchased from Chem-Impex International. Glucose-6-phosphate dehydrogenase (G6PD) was purchased from Worthington Biochemical Corporation. Whole sheep blood was purchased from Innovative Research. Pullulan (desalinized, MW ~200 kDa) was purchased from Polysciences Inc. All other standard laboratory reagents were purchased from Millipore-Sigma. Water used in this study was deionized using a Millipore Advantage A10 purification system to a resistance of 18.2 MΩ cm.

Biosensor fabrication

A scheme outlining biosensor fabrication, sample preparation and the test procedure is shown in Fig. 2A and B, respectively. A wax printer (Xerox ColorQube 8580) was used to print a yellow and black hydrophobic wax barrier pattern on Whatman #1 chromatography paper that was previously laminated on one side using a Scotch™ Thermal Laminator. After printing, the paper was heated in an oven at 120 °C for 2–3 minutes to melt the wax through the thickness of the paper, generating an effective hydrophobic barrier. The paper was then cut into multiple strips which displayed the final colorimetric output for both a control (C) spot to discern color resulting only from the blood itself, and a test (T) spot which

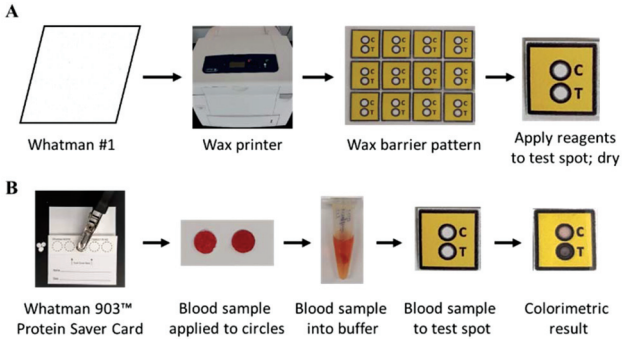

Fig. 2 (A) Preparation of paper biosensor. (B) Sample preparation and colorimetric output for G6PD detection.

contained assay reagents. The assay reagent mixture contained: 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. This solution (4 μl) was deposited by pipetting into the "test" spots of the biosensor and air dried for at least 4 hours before use or storage in a sealable bag in the dark.

G6PD detection in whole sheep blood

Whole sheep blood (which tests as G6PD deficient in simple colorimetric assays)[15,16] collected with K_2-EDTA as the anticoagulant[2] was stored at 4 °C and used as soon as possible after being received. A singe lot of whole sheep blood was used in all final quantitative experiments to avoid any variation in hemoglobin levels. Aliquots of blood (100 μl) were spiked with G6PD at various levels according to the WHO G6PD classification (see Table S-1†) to obtain blood samples representing deficient (4 U dL^{-1}), intermediate (60 U dL^{-1}), normal (132 U dL^{-1}) and above normal (220 U dL^{-1}) G6PD concentrations. A volume of 15 μl of each G6PD-spiked blood aliquot was applied within a 5 mm cut-out sample circle (standard single hole punch) from a Whatman 903 Protein Saver™ card. The paper was immediately transferred into separate 1.5 ml microfuge tubes containing 1 ml of 12.2 mM (5 mg ml^{-1}) MTT in 50 mM Tris pH 7.5 (MTT buffer solution) and incubated at ambient laboratory conditions (20 ± 2 °C, ~40% relative humidity (RH)) for 10 minutes during which time the blood partially eluted into the MTT buffer solution. The samples were mixed three times by inversion before 5 μl was applied to the control and test spots on the paper biosensor. The resulting color change was observed until the samples air dried naturally, normally within ~40 minutes. Assays were performed at least in triplicate on separate days.

G6PD detection at elevated temperature and humidity

The G6PD detection assay was carried out at various temperatures and levels of humidity using an Environmental Chamber (Caron) set to: 21 °C/75% RH, 37 °C/40% RH and 37 °C/75% RH. Test spots were prepared with the assay reagent mixture the day before and stored overnight covered with foil at 20 °C, 40% RH. The MTT buffer solutions and G6PD-spiked blood

samples were prepared the day of the test. The MTT buffer solutions were incubated at the test temperature for 30 minutes prior to use. The entire detection assay was performed at the designated temperature and humidity.

Quantitative image-based analysis

For development and optimization of the assay conditions, images of individual triplicate G6PD detection assay results were taken over the course of the assay or at the end-point (when samples had just dried) using the colorimetric blot setting on a ChemiDoc MP imaging system (Biorad) with the Blot/UV/Stain-free gel tray using automatic signal intensity; the grey-scale image color was inverted. Fiji[17] was used to quantify the intensity of the sample spots from the image. The background intensity (0 U dL^{-1}) was subtracted from the value for all other G6PD concentrations on the same test strip. All replicates for each G6PD concentration were averaged and represented graphically.

Visual analysis by trained user

The G6PD detection assay was carried out at room temperature using 12 enzyme-spiked blood samples, prepared and recorded by a colleague not involved in the study, on two separate days (24 tests in total). Visual analysis and assignment of G6PD levels (deficient, intermediate, normal or above) was carried out blindly by a trained user without any assistance (*e.g.*, no color card was used) or knowledge of the G6PD level present in the prepared samples. Predicted *versus* actual designations were compared.

Visual analysis by untrained users

Following blind testing by a trained user, a color card was generated to assist with differentiation of G6PD designations and thus avoid the need for a reader. The HEX color value was recorded for each color averaged replicate spot for each G6PD level (deficient, intermediate, normal and above). Using a color converter (WorkWithColor.com), the HEX code was translated to CIE $L \times a \times b$ values. These values were averaged for the replicates of each G6PD concentration and grouped according to the G6PD designations. The averaged CIE $L \times a \times b$ values were then converted to RGB values to prepare the color card, which had four colors, each corresponding to a different G6PD designation. Twenty assays covering the four G6PD designations were prepared in random order by a trained user then visually assessed *via* comparison to the color card (by 4 untrained users) or the color card and a sample assay (by 3 different untrained users) who then assigned a predicted G6PD designation for each assay. Predicted *versus* actual designations were compared.

Results and discussion

G6PD deficiency can lead to obvious clinical signs including fatigue, rapid heart rate, shortness of breath and jaundice.[18] However, due to gender variations (heterozygosity in females[19]), genetic diversity (215 mutations[20]), geographic location[21] and population specific median red blood cell counts,[22] many people – mostly heterozygous females – fall into the intermediate range (WHO Class III), which is difficult to distinguish from normal and deficient using the current binary rapid diagnostic tests (RDT). Additionally, these dissimilarities make it difficult to define singular classification cut-off values to incorporate into POC devices.[22–24] As a result, definitive testing for G6PD levels currently requires centralized testing in a laboratory by trained personnel.

Initial efforts to develop a semi-quantitative, facile POC paper biosensor to detect G6PD deficiency from whole blood required significant optimization of paper biosensor design, color output, reagent concentrations and stability, and sample preparation. We selected 1-PMS as the electron carrier and MTT as the optimal chromogenic reagent (Fig. S-1†). Concentrations of all assay reagents were optimized to produce the largest color change on paper sensors (denoted as Mix #6 in Table S-2 and Fig. S-2†). Based on our past reports on stabilization of reagents in pullulan,[25–28] all reagents in Mix #6 were combined with 5% (w/v) pullulan to ensure stability and functionality of the reagents once dried on paper. However, we observed that MTT spontaneously converted to its formazan dye within a few days when present with the Tris component of the assay mixture (see Fig. S-3,† top), while MTT alone in water was stable for much longer (see Fig. S-3,† bottom). MTT was tested a dried reagent on a separate paper component that was added to the blood sample but did not result in a robust or consistent assay output (data not shown), so solid MTT was dissolved in the blood dilution buffer (50 mM Tris, pH 7.5) just prior to use rather than being present directly on the sensor or as an accessory module.

Based on preliminary testing in solution (Fig. S-4 to S-6†), it was determined that 1 : 100 v/v of blood sample added to the MTT buffer solution was optimal for color differentiation (Fig. S-4†). When the assay was moved initially to paper, a larger reagent volume (5 ml MTT buffer solution, Fig. S-5†) and blood sample volume (60–75 μl, Fig. S-6†) were used. In the interest of reducing both inputs for cost effectiveness and patient comfort, respectively, the assay was scaled down to 1 ml of MTT buffer solution and 15 μl of blood spotted onto a smaller piece of Whatman 903 card; assay performance was maintained at this scale.

The final test procedure required four steps: (1) blood collection on a Whatman 903 Protein Saver™ card; (2) elution of the blood sample into a freshly prepared MTT buffer solution; (3) application of 5 μL of the buffered blood solution to the optimized paper biosensor (Fig. 2B); and (4) evaluation of the color change of the sample over time. The yellow wax barrier pattern was chosen as it contrasted well with the brown output color of the assay (a combination of the natural purple color of MTT and the dark red blood). The empty control spot (with no reagents) provided assurance that the color observed in the test spot was not due to the blood sample itself; the control spots dried to the color of a deficient blood sample regardless of G6PD concentration (Fig. 2B, colorimetric result). We opted

to avoid a lateral flow design since this tends to smear and ultimately dilute the color signal along the flow path, a noted annoyance with both the Alere BinaxNOW® G6PD test and the Access Bio CareStart™ G6PD test.[7] As shown in Fig. 3A, restricting the enzymatic reaction within a small area increased color intensity. Initial testing showed variability in the color intensity between samples with the same G6PD concentration when the buffered blood solution was not mixed thoroughly just prior to application to the paper biosensor, or when the drying rate of the samples differed owing to day-to-day changes in the ambient relative humidity (RH). The former issue was easily solved with thorough mixing of the sample prior to use. RH could be controlled with a simple water saturated container, which would allow constant RH even if the assay was performed in an arid resource limited region.

As seen in Fig. 3A, the color intensity of G6PD-spiked blood samples followed a trend of darkening brown color as the concentration of G6PD was increased from deficient to above normal levels. Fig. 3B shows distinct differences in image intensity between deficient, intermediate, normal and above normal G6PD levels, demonstrating a superior ability to differentiate the four G6PD designations relative to visual detection of color changes.

A noted drawback of off-site blood testing is the need to store samples at 4 °C, and even then, enzyme activity drops by >10% after 9 days[29] and is drastically reduced after 14–21 days.[30] With the paper biosensor, as well as the current RDTs, blood can be taken from a finger prick and tested immediately. The advantage of the paper biosensor is that it does not require precise measurement of blood sample volumes. The only requisite is to soak the Whatman 903™ Protein Saver card circle with sample; at 5 mm diameter, the test simply requires 10 μl or more of blood so as to fill the sample card circle and generate the desired assay response (see Fig. 4). This use of a blood collection card was chosen for its simplicity and familiarity,[31] as it is employed for a range of clinical analyses from dried blood spots.[32]

Given that the prevalent geographical areas of G6PD deficiency are tropical, having a test that is functional at high temperature and humidity is desirable. The assay was tested at 21 °C and 37 °C, and at both 40% and 75% RH (see Fig. 5). Surprisingly, temperature and humidity didn't affect the assay significantly; only the above normal G6PD concentrations were significantly altered. As expected, we observed that the sample

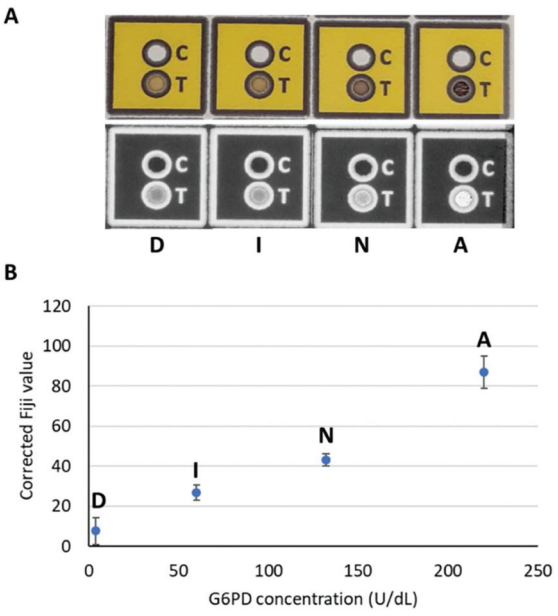

Fig. 3 (A) Detection of G6PD levels in blood corresponding to each WHO classification, showing a typical assay at 21 °C, 40% relative humidity with the original color output (top) and after imaging using a ChemiDoc MP imaging system (bottom). (B) Average color intensity of 12 replicates of each G6PD designation measured from ChemiDoc MP grey-scale images using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal). Data represents triplicate experiments on four separate days.

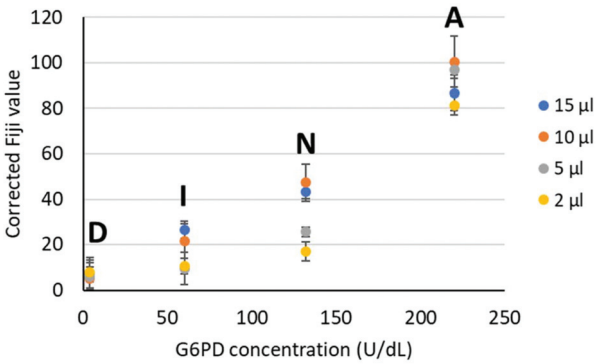

Fig. 4 G6PD detection with varying blood volumes applied to the sample card at 21 °C, 40% relative humidity. The average color intensity of triplicate test spots at each G6PD designation was measured from grey-scale ChemiDoc MP images using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal).

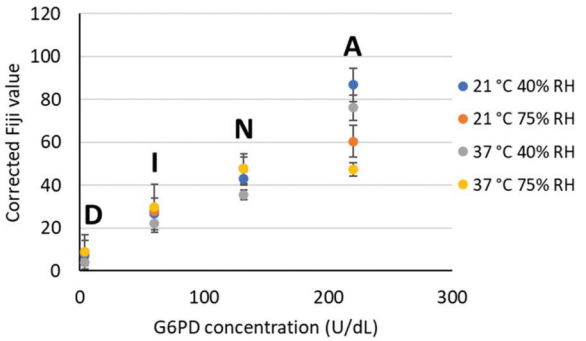

Fig. 5 G6PD detection in blood at higher temperature and/or humidity. The average color intensity of triplicate test spots at each G6PD designation was measured from grey-scale ChemiDoc MP images using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal). Data for 21 °C, 40% RH represents 12 replicates; remaining data represents triplicate experiments.

dried faster at 40% RH and that the enzymatic reaction proceeded faster at 37 °C. At 42 °C, only normal and above normal samples generated color since the samples dried too rapidly (<10 minutes), providing insufficient reaction time for full color development (data not shown).

The time needed for sample elution into the MTT buffer solution was also considered as it would be an important aspect of a future POC test. We found that assay outcomes after elution times of 5–20 minutes were nearly identical (Fig. S-7†). This suggests that the amount of G6PD eluted from the card is reproducible, and would facilitate development of a rapid test, allowing some flexibility in the timing of the sample preparation by the user.

A small set of paper biosensors were prepared for long-term stability testing; they were stored in a dark cupboard at ambient temperature and humidity and tested over a period of six weeks. The results indicated excellent reagent stability, with color development identical to that seen when the MTT buffer solution and biosensors were prepared and used the same day (Fig. S-8†).

We also confirmed that the colorimetric change we observed in the assay was due to G6PD and not the downstream pentose phosphate pathway enzyme 6-phosphogluconate dehydrogenase (Fig. S-9†).

To more practically assess sensor performance, we utilized visual testing of random enzyme-spiked whole sheep blood samples (Fig. 6A). As expected, the above normal and deficient G6PD samples were easy to determine by eye, even without a color card, while the smaller difference between intermediate and normal samples was more difficult to predict reliably, even by a trained user (identification accuracy of 82% by the trained user, without visual cues, in the random test) (Fig. S-10†).

To aid in visual detection, a color card was generated (Fig. 6B, top) to allow more accurate assessment of color intensity for proper assignment of G6PD designations by untrained users. Twenty G6PD assays were prepared by a trained user using known concentrations of G6PD and then assessed by untrained evaluators (Table S-3†). In the first assessment, four evaluators had only a color card (Users #1–4); in a second assessment, 3 different evaluators (Users #5–7) had the color card along with a complete detection assay (Fig. 6B, bottom). Using only the color card, untrained users accurately assigned 14/20 G6PD designations on average; with the color card and complete assay, untrained users correctly assessed ~17/20 G6PD designations (Table S-3†). Specific errors made differentiating between deficient/intermediate, intermediate/normal and normal/above are highlighted in Table S-4.† Misidentification of deficient/intermediate or normal/above would have no effect on patient diagnosis/treatment. The challenge lies in consistently distinguishing intermediate from normal.

One drawback to the developed assay was the time needed for the samples to dry, which was necessary to distinguish a difference between normal and intermediate or deficient samples. Given that temperature and humidity factor into the time for the sample to reach this end-point, and that this end-point could take up to 60 minutes, we looked more closely at the color changes occurring within the first 30 minutes of the assay while the samples were still wet. As shown in Fig. 7, a time-based readout method (*i.e.*, time required to reach a specific color intensity) could be used to determine a patient's G6PD status much more quickly. Above average samples pro-

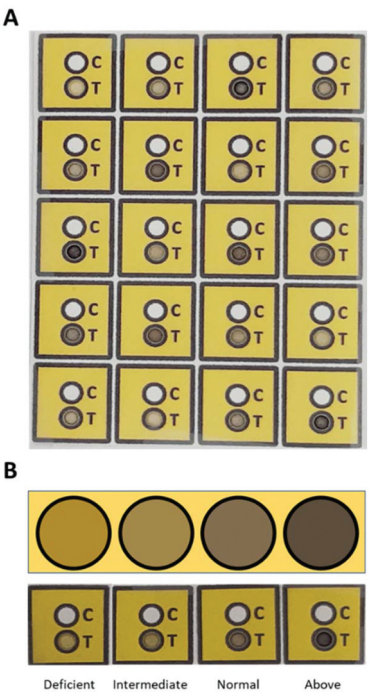

Fig. 6 (A) A sample of random G6PD detection assays for assessment by untrained users. (B) (Top) Color card to assist in identification of individual G6PD designations. (Bottom) Expected assay result provided to untrained users to help assess G6PD designations.

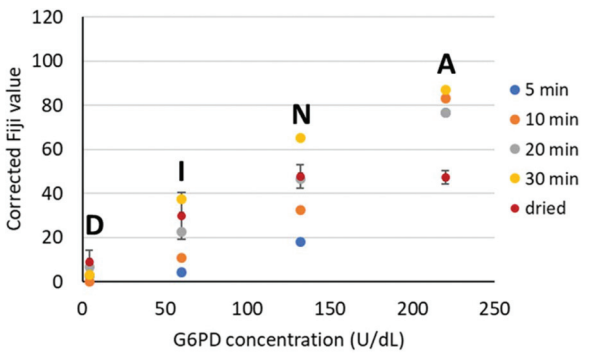

Fig. 7 G6PD detection in blood as a function of time after initiating the assay (5–30 minutes) *versus* use of dry sample (on average, 40 minutes) at 21 °C, 40% relative humidity. The average color intensity of replicate test spots (where indicated by standard error bars) of each G6PD designation was measured from grey-scale ChemiDoc MP images using Fiji (D = Deficient; I = Intermediate; N = Normal; A = Above normal). Note that the data point for 5 and 20 minutes in the "Above normal" samples overlap. Samples for 5–30 minutes were triplicates; dried samples represent 12 replicates.

Table 1 Comparison with two current rapid diagnostic tests for G6PD deficiency

	Blood sample	Temp range	Cut off (U dL^{-1})	Color output	Time to result	Cost (USD)
BinaxNOW®	10 μl	18–25 °C	~60	Purple	5–7 min	$25
CareStart™	2 μl	20–32 °C	~40	Purple	10 min	$1.50
Paper sensor	15 μl	20–37 °C	~60	Brown	5–40 min	$3

duced an obvious color change within one minute, normal samples darkened within 5 minutes, intermediate samples required ~10 minutes to generate color, while deficient samples required up to 20 minutes before the start of any color change. Based on this, samples that required longer than 5 minutes to show any color development would suggest that G6PD concentrations are low enough to warrant referral to proper laboratory quantitative testing before beginning any type of treatment.

In general, RDT/POC devices for the detection of any medically relevant infliction strive to fulfill the ASSURED criteria released in 2006 by the WHO:[33] **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid/robust, **E**quipment-free, and **D**eliverable. Under these guidelines, this proof-of-concept paper biosensor meets most of these criteria. The test is affordable (under $3 USD per test using list prices from major manufacturers; see Table 1), sensitive with few false negatives, specific with minimal false positives, user-friendly, relatively rapid, can be made equipment-free and is sufficiently stable to be deliverable to remote locations.

Recently, Drain *et al.*[34] proposed new criteria for POC tests with emphasis on the immediacy of treatment, infrastructure (no need for equipment) and true cost (wages, transport, storage) of the tests where they are most commonly used. The suggested criteria are: Expedited Clinical decision, Use at the point-of-care, Affordable, Rapid, Acceptable test efficacy, and Cost-effective. Again, the prototype G6PD test meets these alternate criteria, as it could enable expedited clinical decisions with reasonable efficacy (80% accuracy) while remaining cost effective, as there is no need for a cold-chain for transportation or refrigeration to store samples off-site or to use expensive equipment to interpret the test.

The G6PD paper biosensor reported here, though still an early prototype, is comparable to the G6PD RDTs currently available (see Table 1) but has some key advantages: there is no need to measure the initial volume of blood upon sampling; the test can operate over a higher temperature range; accurate delivery of the small volume of diluted blood sample onto the test spot of the biosensor can be facilitated easily by including a metered transfer pipette; and better discernment between all G6PD designations is possible by including a color card and/or completed assay sample, or by a using time-based readout method as described above.

In addition to the commercially available RDTs for detection of G6PD deficiency, a device created by the PATH Diagnostics Group was described by Kahn *et al.*,[35] which also aimed to distinguish between normal, intermediate and deficient levels of G6PD. Their process required four steps, a lateral flow device and a hand-held reader to determine the resulting color intensity, which may not be easily discernible by eye. Our approach replaces the reading device with a simple color card/assay and achieves comparable distinction between normal and below normal G6PD concentrations. In addition, our paper-based test can be used with diluted whole blood deposited directly onto the device, while the PATH device required removal of red blood cells prior to running the test. The challenges faced by all researchers aiming to provide a reliable POC G6PD test was recently articulated by Pal *et al.*;[36] creating a robust device that can be used by untrained care providers that meets the required enzyme activity threshold cut-offs in all populations and environments remains a considerable task.

Conclusions

A semi-quantitative paper-based proof-of-concept POC biosensor was developed using stabilized reagents on a paper-based device to allow for rapid colorimetric screening of G6PD levels in a simple and rapid manner that is amenable to resource-limited settings. The paper biosensor presented here has a simple design with all key reagents included either on the paper or in the diluent buffer, thereby reducing manipulation steps by the user. In addition, the test is compatible with currently used sample collection and processing methods and can provide a greater detection range of G6PD levels beyond the "yes/no" outputs of currently available tests. An important aspect of the current test format is that it is universally applicable to any reaction involving a dehydrogenase, as these can all be linked to colorimetric detection of a formazan dye product using β-nicotinamide adenine dinucleotide (NAD) or β-nicotinamide adenine dinucleotide phosphate (NADP) as a coenzyme. As such, it should allow detection of enzymes, substrates or enzyme inhibitors using a common platform. Additional examples of paper-based coupled enzyme assays will be reported in future manuscripts.

Conflicts of interest

There are no conflicts to declare.

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