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BACKGROUND. Anemia, or low blood hemoglobin (Hgb) levels, afflicts 2 billion people worldwide. Currently, Hgb levels are typically measured from blood samples using hematology analyzers, which are housed in hospitals, clinics, or commercial laboratories and require skilled technicians to operate. A reliable, inexpensive point-of-care (POC) Hgb test would enable cost-effective anemia screening and chronically anemic patients to self-monitor their disease. We present a rapid, stand-alone, and disposable POC anemia test that, via a single drop of blood, outputs color-based visual results that correlate with Hgb levels.

METHODS. We tested blood from 238 pediatric and adult patients with anemia of varying degrees and etiologies and compared hematology analyzer Hgb levels with POC Hgb levels, which were estimated via visual interpretation using a color scale and an optional smartphone app for automated analysis.

RESULTS. POC Hgb levels correlated with hematology analyzer Hgb levels (r = 0.864 and r = 0.856 for visual interpretation and smartphone app, respectively), and both POC test methods yielded comparable sensitivity and specificity for detecting any anemia (n = 178) (<11 g/dl) (sensitivity: 90.2% and 91.1%, specificity: 83.7% and 79.2%, respectively) and severe anemia (n = 10) (<7 g/dl) (sensitivity: 90.0% and 100%, specificity: 94.6% and 93.9%, respectively).

CONCLUSIONS. These results demonstrate the feasibility of this POC color-based diagnostic test for self-screening/self-monitoring of anemia.

TRIAL REGISTRATION. Not applicable.

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Introduction

Anemia, defined by low hemoglobin (Hgb) concentrations in the blood, affects one-third of the world’s population, approximately 2 billion people, and is especially prevalent in young children, women of childbearing age, and the elderly (1). Symptoms of anemia range from weakness, fatigue, and dizziness in milder cases to life-threatening cardiovascular collapse in more severe cases, and chronic anemia leads to permanent neurocognitive deficits in children (2, 3). The most common causes of anemia are nutritional deficiencies (e.g., iron deficiency), primary hematologic diseases (e.g., sickle cell disease, thalassemia, myelodysplastic syndromes), drugs (e.g., cancer chemotherapy), and other chronic conditions that indirectly cause anemia (e.g., chronic kidney disease, inflammatory/autoimmune disorders, HIV) (4–7). Even in developed nations, anemia remains a major public health issue, as an estimated 85 million people are at high risk for anemia and 5 million people are afflicted by anemia, respectively, in the US alone (8–11).

Currently, the gold standard for anemia diagnosis is a complete blood count (CBC) using a hematology analyzer, which measures the Hgb levels of a venous blood sample. As CBCs require a venous blood draw from a trained phlebotomist and hematologY
Therefore, the device is feasible as a home-based test for self-screening or self-monitoring of anemia. Each test requires a single 5 μl drop of capillary blood obtained via finger stick and can be interpreted with the naked eye. A clinical assessment study was conducted using blood samples from patients with anemia of different degrees and etiologies comparing visual interpretation of our POC test results by untrained users with clinical CBC Hgb levels obtained simultaneously. In addition, we present results from an optional custom color analysis smartphone app that integrates with the POC anemia test for automated Hgb level measurements and potential data transmission.

Results

Our color-based POC anemia test differentiates among different degrees of anemia with a visual readout from a quick, inexpensive, disposable, and stand-alone point-of-care (POC) anemia diagnostic that is simple enough for untrained people to use as a self-test will enable (a) patients with chronic anemia to self-monitor their disease, improve their quality of life, and potentially improve clinical outcomes and (b) the general public to self-screen for anemia in a cost-effective manner. While several POC anemia diagnostics are currently available, all are currently designed for use by healthcare professionals and none are designed for home use as a self-test (12–16).

To those ends, here we present a visual color-based POC anemia test. The assay is rapid (<60 s), simple to use, disposable, and inexpensive and is a stand-alone system that does not require electrical power, additional equipment, or significant training.
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Figure 2. Quantification of POC color-based anemia test results. (A) Spectrophotometric absorbance curves for resultant solution colors for blood samples with Hgb levels of 8.0 g/dl, 9.1 g/dl, 10.5 g/dl, 11.6 g/dl, and 16.1 g/dl (λ = 450–700 nm, 5-nm step size). Absorbance maxima for the heme peaks are at 540 nm and 575 nm, and the maximum for the TMB peak is at 630 nm. (B) Standard curve was created using a single blood sample diluted in native plasma to create Hgb levels ranging between 5.5 g/dl and 17.2 g/dl. The final a* value, the coordinate in the CIE L*a*b space representing the balance of red-green color in each resultant solution, is plotted against Hgb level, as measured with a standard hematology analyzer. R value is 0.997.

Numerous tests and measurements were carried out to ensure the reliability and accuracy of the POC test. In Figure 2A, spectrophotometric absorbance curves are shown for blood samples with different Hgb levels, ranging from 8.0 g/dl to 16.1 g/dl. The absorbance maxima for the heme peaks are at 540 nm and 575 nm, and the maximum for the TMB peak is at 630 nm. A standard curve was created by diluting a single blood sample with native plasma to obtain samples with Hgb levels ranging from 5.5 g/dl to 17.2 g/dl. The final a* value, the coordinate in the CIE L*a*b space, is plotted against Hgb level, as measured with a standard hematology analyzer. The correlation coefficient (R) is 0.997.

Mechanistically, the assay involves a modification of the reaction among Hgb, hydrogen peroxide, and 3,3′,5,5′-tetramethylbenzidine (TMB), in which Hgb catalyzes a redox reaction between TMB and hydrogen peroxide, leading to oxidized TMB products that can be detected colorimetrically (see Supplemental Figure 1 and ref. 18). This reaction has been well characterized (18–21) and has been used commercially for spectrophotometric detection of Hgb in plasma, qualitative detection of blood in stool samples, and crime scene investigation and in portable single-wavelength photometers for POC anemia testing (22, 23). Our group has repurposed and modified this reaction to (a) yield differential product ratios that exhibit different colorimetric properties spanning the visual spectrum, correlating with clinically relevant Hgb levels in whole blood, and (b) improve reagent stability and shelf-life. These novel modifications allow for the translation of this assay into a POC, disposable, visual color-based test for screening and monitoring anemia that does not require any additional spectrophotometric equipment or electrical power.

Standard spectrophotometry was used to quantitatively analyze colorimetric aspects of our assay. Absorbance curves (λ = 350 nm to 750 nm) were obtained for whole blood samples of different Hgb levels, ranging from 8.0 g/dl to 16.1 g/dl (Figure 2A). Upon completion of the redox reaction, blood samples with different Hgb levels yielded different resultant colors and, as expected, were associated with characteristic absorbance curves. Absorbance curves revealed different maxima for each resultant color: red-colored results (normal Hgb levels) are characterized by maxima at 540 nm and 575 nm, indicating the presence of heme, whereas yellow-colored results (mild to moderate anemia) are characterized by a maximum at 450 nm and blue- and green-colored results (severe anemia) are characterized by a maximum at 630 nm, indicating the presence of oxidized TMB. To enable direct quantitative comparisons with blood Hgb levels, the absorbance spectra are converted into single values, which can be accomplished by measuring the absorbance ratios at selected wavelengths or by converting the spectra into coordinates of color space systems, such as the CIE L’/a’/b’ space (24, 25). Color analysis revealed that our absorbance data were primarily dependent on the red-green axis of the CIE L’/a’/b’ space and independent of the other axes. As such, a standard curve was then created to relate Hgb levels with quantified color using a* values, the red-green axis CIE L’/a’/b’ coordinates, which were converted from the absorbance data of the resultant colors of the POC anemia test (Figure 2B and ref. 23).

For these experiments, blood obtained from healthy donors was diluted with native plasma to obtain samples with Hgb levels ranging from 5.5 g/dl and 17.2 g/dl, and our POC anemia test was run on each sample in quadruplicate. As shown, a second-order polynomial fit indicates a strong correlation between resultant POC test color and Hgb level (r = 0.997).

A clinical assessment comparing our POC anemia test with a standard hematology analyzer was performed using blood samples obtained from patients (n = 238; see Supplemental Table 1 for a list of patient diagnoses) with varying degrees of anemia and etiologies thereof and consisted of absorbance data, visual interpretation by untrained users, and a colorimetric smartphone app for automated analysis. As an objective measure of the accuracy of our assay, absorbance data (λ = 350 nm to 750 nm) were obtained on a well plate reader. In addition, digital images of the resultant POC solutions were obtained via a standard smartphone camera under ambient indoor lighting conditions. To assess for assay variability, each clinical sample was tested in quadruplicate using 4 separate POC anemia tests. Supplemental Figure 2 depicts clinical patient results using our POC test (n = 238), as analyzed with a* value versus clinical hematology analyzer Hgb levels. The average of the standard deviations of the separate POC test results for each clinical sample was 1.009 in a* value, corresponding to 0.20 g/dl Hgb, establishing the inter-test variability of our POC anemia test.

Visual estimates of the POC anemia test results versus clinical hematology analyzer Hgb levels from patient samples (n = 238) is presented in Figure 3. This aspect of the clinical assessment specifically involved 12 untrained adult subjects, with no known diagnosis of color blindness, who interpreted visual data and assessed a survey of the images of POC anemia test results that were obtained for each clinical sample. This usage of images allows for the assessment of inter-user variability, as each subject will view...
As a potential home diagnostic test, we also tested the capability of our POC anemia test for serial Hgb level monitoring. Figure 5 depicts POC color-based results over 45 days for a healthy control male, a healthy control female, and a male with β-thalassemia major, a genetic disease that causes chronic anemia, who receives monthly red blood cell transfusions. Absorbance ratios (540 nm/630 nm) were used to estimate Hgb levels, which were in agreement with the subjects’ corresponding Hgb levels obtained via hematology analyzer obtained the same days as the POC anemia tests were performed (Figure 5). As shown, the Hgb levels of the control male do not show any significant deviation from baseline throughout the course of the study. In contrast, the Hgb levels of the female control indicate lower Hgb at days 15 and 41, which coincided with menstruation. The Hgb levels of the thalassemic subject were low on days 3, 27, and 41, which coincided with the days just prior to red cell transfusion. Overall, the color-based POC test was able to accurately track distinguishable Hgb level changes over time.

Discussion

This color-based POC anemia test offers rapid, simple, inexpensive, and disposable self-screening and self-monitoring for anemia at home, requiring only a finger stick drop of capillary blood. The key unique aspects are its (a) visual color-based readout, enabling its use as a stand-alone system that does not require electrical power or additional equipment (such as a single-wavelength photometer); (b) low cost (each device currently costs US $0.50 to manufacture and is disposable); (c) small size and portability; and (d) ease of use, which enables users and patients to test themselves at their convenience. We have also developed an optional colorimetric smartphone app, with which the user simply takes an image of the resultant color change of the POC anemia test and the app outputs an estimated Hgb level on the smartphone screen. While not a requirement, this app provides automated analysis, with the built-in color scale sticker for users who are color-blind or prefer to not use their own judgment in estimating Hgb levels.

A clinical assessment determined that the POC anemia test obtained similar results to those of hematology analyzers ($r = 0.864$ and $r = 0.856$ for visual interpretation and smartphone app, respectively), and both POC test analysis methods yielded comparable sensitivity and specificity for detecting anemia ($<11$ g/dl) (sensitivity: 90.2% and 91.1%, specificity: 83.7% and 79.2%, respectively) and severe anemia ($<7$ g/dl) (sensitivity: 90.0% and 100.0%, specificity: 94.6% and 93.9%, respectively). These values are similar to those of existing POC anemia diagnostic tests, which currently are not designed for, and are not practical for, home use or use as a self-test (12–17, 26, 27). These data also highlight the

### Table 1. Sensitivity and specificity via visual interpretation

<table>
<thead>
<tr>
<th>Anemia Type</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe anemia (&lt;7 g/dl)</td>
<td>90.0%</td>
<td>94.6%</td>
</tr>
<tr>
<td>Mild anemia (&lt;11 g/dl)</td>
<td>90.2%</td>
<td>83.7%</td>
</tr>
</tbody>
</table>

Sensitivity and specificity of POC estimated Hgb via visual interpretation were calculated for detecting severe anemia (<7 g/dl) and the presence of any anemia (<11 g/dl).
anemia and seek medical attention when their Hgb levels decrease significantly from their baseline (9–11, 28). Indeed, chronic hemolytic anemia patients could potentially use this system for early self-detection of worsening anemia, which could be sudden, rapid, and potentially life threatening, in the context of viral infections or onset of acute complications in the case of sickle cell disease. Finally, as our POC anemia test requires a small amount of blood (5 μl) from a simple finger stick, this diagnostic would be especially useful to minimize blood loss due to laboratory testing in neonates and children, especially if Hgb levels need to be checked repeatedly (26).

In addition, our current chemical formulation yields color results correlating to different levels of anemia, as defined by the World Health Organization, <7 g/dl for severe anemia and <11 g/dl for mild anemia (29). This chemical formulation can be altered in a finely tuned manner to “shift” the color scale to correspond to different ranges of Hgb levels (Supplemental Figure 4). As shown, the resultant solution colors of the current chemical formulation accurately differentiate between Hgb levels of 5.5 g/dl and 17.2 g/dl. The left-shifted curve depicts another formulation of the POC anemia test that can produce different color results corresponding to Hgb levels ranging from 2.5 g/dl to 9.1 g/dl and would be especially useful for diagnosing patients with severe anemia in the global health and low-resource settings. In contrast, the right-shifted curve depicts another formulation designed to detect and differentiate Hgb levels ranging from 13.6 g/dl to 25.4 g/dl, and, as such, this formulation can be used to accurately diagnose the mildest forms of anemia as well as differing degrees of polycythemia. Therefore, the exact formulation of the POC anemia test will be dictated by the disease type and clinical scenario.

While several devices are currently available for POC anemia testing, the vast majority of these systems remain in the clinical setting, due to high costs and required technical skill for use, in comparison with our POC test. For example, portable POC devices that measure Hgb levels exist, but they are not stand-alone, consisting of an electrically powered analyzer and disposable cartridges, and also require technical training (13, 26). The majority of these analyzers cost between US $1,000 and $2,000, with each test itself costing US $1–2 thereafter. More recently, noninvasive co-oximeters offering continuous Hgb level measurements have been developed, but they cost between US $1,000 and $3,000 and also require electrical power and technical training (17, 22). In an attempt to introduce a simple, low-cost alternative for low-resource settings, the World Health Organization developed the Haemoglobin Colour Scale to test for anemia using the color of one’s blood to differentiate between Hgb levels, such that anemic samples reflected a paler red color as compared with that of normal samples (12–16). While initial results indicated acceptable
all, our results demonstrate the feasibility of this POC anemia test to function as a self-screening and self-monitoring tool for any patient who has or is at risk for anemia.

**Methods**

*Color-based POC anemia testing.* Each color-based POC anemia device was prefilled with 500 μl of a modified TMB and reagent solution and was coupled with a 5-μl straight, sodium heparin-coated, capillary tube (Innovative Med Tech) for blood collection. Blood sample preparation for standard calibration curves experiments (Figure 2) and stability testing involved obtaining blood from a healthy subject via venipuncture procedure into a heparinized container. Blood was then centrifuged at 150 g for 10 minutes to separate plasma from red blood cells. Different dilutions (ranging from 6.8 g/dl to 17 g/dl, as indicated) were prepared by mixing different ratios of native red blood cells and platelet poor plasma. Hgb levels were determined via an Advia 2120 Hematology System. All device prototypes were fabricated in the Global Center for Medicine Innovation.

*Obtaining and processing patient blood samples.* All patients enrolled in our study obtained a CBC for their clinical care the same day, typically immediately before, blood was obtained for the color-based POC anemia test. Patient samples were obtained, and CBCs, which include a Hgb level, were run on standard clinical grade hematology analyzers (Advia 2120i, Siemens Inc. and Heska Hematrace, Heska). Patient blood samples for the color-based POC anemia test were collected via finger stick with a spring-loaded lancing device (One Touch Ultra Soft) and a disposable 21-gauge lancet. As quadruplicate measurements using the color-based POC anemia test were conducted for each sample (n = 4), 100–200 μl capillary blood was collected from each patient subject. Once the assay reaction was complete, equal portions of the solution were transferred into 4 wells of a 96-well plate. Absorbance values across the visual spectrum (λ = 350 nm to 750 nm, 5-nm step size) were then recorded using a well plate spectrophotometer (SpectraMax M2/M2e).

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**Table 2. Sensitivity and specificity via smartphone app**

<table>
<thead>
<tr>
<th>Anemia Level</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe anemia (&lt;7 g/dl)</td>
<td>100%</td>
<td>93.9%</td>
</tr>
<tr>
<td>Mild anemia (&lt;11 g/dl)</td>
<td>91%</td>
<td>79.2%</td>
</tr>
</tbody>
</table>

Sensitivity and specificity of POC estimated Hgb via smartphone app were calculated for detecting severe anemia (<7 g/dl) and the presence of any anemia (< 11 g/dl).
Overall, 238 clinical blood samples from pediatric and adult patients were collected, tested, and analyzed using our POC anemia test and were compared with the patients’ clinical CBC results. There were no adverse effects experienced by study subjects. In addition, one healthy adult male, one healthy adult female, and one male with β-thalassemia major were recruited for serial testing over 45 days (n = 1 data point per subject per day tested). For this serial study, each sample was tested using our POC anemia test and a hematology analyzer, either an Advia 2120 Hematology System or a Heska Hematrue analyzer (shown to correlate with the Advia system, r = 0.998). The research technician was blinded to golden standard CBC results until testing was complete. Each sample was tested in quadruplicate (n = 4) using the POC anemia test.

**Assay color quantification via a* value analysis.** To enable head-to-head comparison of the color-based POC anemia test with Hgb levels obtained from a standard clinical hematology analyzer, the color changes the reagent undergoes must be quantified and distilled to a single number. One method commonly used in spectroscopy is to take the ratio of 2 absorbance intensity peaks, as shown in Figure 2A. Another method involves conversion of absorbance data into CIELAB L*a*b* space data (24). Absorbance data across the visual spectrum (λ = 350 nm to 750 nm, 5-nm step size) were obtained with a well plate spectrophotometer (SpectraMax M2/M2e) and imported into MATLAB. The code analyzed color as a value of a* and correlated values to Hgb levels using the procedure described below.

Colors correspond to the CIE L*a*b* (CIELAB) color space. The CIELAB space is a tricordinate color space, wherein the L*, a*, and b* coordinates correspond to the white-black, red-green, and yellow-blue balances of a given color, respectively (24, 25). The CIELAB color space was originally generated to allow for a perceptually uniform color space, hence changes in colors should accompany a change in quantified color value (24). To generate comparable color data in the CIELAB space, absorbance data spanning λ = 350 nm to 750 nm, 5-nm step size, was used to first calculate color values in the CIE 1931 XYZ space, using the equations listed below.

\[
X = K \int_{430}^{670} S(\lambda) \bar{x}(\lambda) R(\lambda) d(\lambda)
\]

(Equation 1)

\[
Y = K \int_{430}^{670} S(\lambda) \bar{y}(\lambda) R(\lambda) d(\lambda)
\]

(Equation 2)

\[
Z = K \int_{430}^{670} S(\lambda) \bar{z}(\lambda) R(\lambda) d(\lambda)
\]

(Equation 3)

\[
K = \frac{100}{\int_{430}^{670} S(\lambda) \bar{y}(\lambda) R(\lambda) d(\lambda)}
\]

(Equation 4)

\(S(\lambda)\) is the relative spectral power distribution of a standard illuminant, in this case a D50 source. \(\bar{x}(\lambda)\), \(\bar{y}(\lambda)\), and \(\bar{z}(\lambda)\), supplied by the CIE, are the 1931 spectral tristimulus values. \(R(\lambda)\) is the spectral reflectance (or transmittance) of the object measured. The values of X, Y, and Z were then used to calculate values for L*, a*, and b* using the following equations:

\[
L^* = 116 \left( \frac{Y}{Y_n} \right)^{1/3} - 16
\]

(Equation 5)

\[
a^* = 500 \left( \frac{X}{X_n} \right)^{1/3} - \left( \frac{Y}{Y_n} \right)^{1/3}
\]

(Equation 6)

\[
b^* = 200 \left( \frac{Y}{Y_n} \right)^{1/3} - \left( \frac{Z}{Z_n} \right)^{1/3}
\]

(Equation 7)

\(X_n, Y_n,\) and \(Z_n\) are the CIE tristimulus of the reference white point.

A script to process these calculations was written in MATLAB. It was noted that the \(L^*\) and \(b^*\) values were largely independent of the Hgb levels. As such, \(a^*\) values, which are dependent on the red-green color balance in each result, correlate to our color scale and, therefore, the Hgb level for each sample (Figure 2 and Supplemental Figure 2).

**Assay color quantification via an optional smartphone app.** Colorimetric analysis was performed using custom software written in MATLAB. A calibration function relating Hgb concentrations to peak histogram values for each RGB channel was used to estimate the Hgb concentration for all sample images. The calibration function was created using images of processed POC anemia devices containing blood samples with known Hgb levels (the same standard calibration results presented in Figure 2B). The center 25% of each calibration image was selected as the region of interest (ROI). RGB values in the ROI were converted to CIE L*a*b* color space using a D50 luminator white point. A histogram for each channel (\(L^*, a^*,\) and \(b^*\)) in the ROI was calculated. Peaks of the channel histograms for each calibration image and corresponding Hgb value were fit using planar regression with the following function: estimated Hgb level = \(w + x^*(L^*_{peak}) + y^*(a^*_{peak}) + z^*(b^*_{peak})\).

This MATLAB function was then converted into a Java-based app for performance on an Android-powered mobile phone. The smartphone app allows the user to take a digital photographic image of the resultant solution color and obtain an estimated digital Hgb level result based on the same function used in MATLAB. After image capture, the app saves the image and outputs an estimated Hgb level on screen (Figure 4A). Using this app, Hgb concentrations were estimated for clinical samples (Figure 4B).

**Visual-based analysis.** Using a standard smartphone (iPhone 4, iOS 6) set to default lighting and focusing, images were obtained of all processed POC anemia tests (the entire device, including the reagent solutions after the reaction was completed) using patient blood samples. These images were then placed in randomized order and compiled into surveys. A blinded study was then conducted in which 12 untrained adult subjects (6 women and 6 men with no known diagnosis of any form of color blindness) were asked to visually interpret and assign Hgb values of the images using a color scale, which was based on colors obtained from POC anemia tests of different but known Hgb levels.

**Statistics.** All statistical analyses were performed using SAS 9.3. A P value of less than 0.05 was considered significant. Pearson correlations and general linear regression models and prediction limits were used to assess associations between Hgb level via hematology analyzer and absorbance ratio, \(a^*\) value, visually interpreted Hgb level, and smartphone app Hgb level. Individual patients were identified.
as either adult or pediatric and investigated for differences in their outcomes via analysis of covariance, controlling for the Hgb level via hematology analyzer covariate. In cases when the assumption of equal regression slopes was violated, linear regression models were run separately for adult and pediatric patients. Sensitivity and specificity results were calculated with both adult and pediatric patients together as well as by considering each patient type individually.

Study approval. Pediatric and adult patient blood samples were obtained in outpatient clinics of the Aflac Cancer and Blood Disorders Center of Children’s Healthcare of Atlanta and Emory’s Winship Cancer Center, respectively, by one consistent research technician using protocols approved by the IRBs of Emory University, Georgia Institute of Technology, and Children’s Healthcare of Atlanta. All samples were acquired after informed consent was obtained from patients or their parents/guardians. Each consented patient only provided one sample on the day of consent.

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