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Measuring Hemoglobin: Point-of-Care Diagnostics for Anemia and Sickle Cell Disease in Low-Resource Settings

by

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ABSTRACT

Measuring Hemoglobin: Point-of-Care Diagnostics for Anemia and Sickle Cell Disease in Low-Resource Settings

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This thesis describes my work to develop point-of-care diagnostic tools to measure hemoglobin concentration and diagnose sickle cell disease.

In an effort to set design parameters for point-of-care hemoglobin analyzers, I assessed the variability of hemoglobin, platelet count, and white blood cell count and differential in successive drops of fingerprick blood. The average percent coefficient of variation in successive drops of fingerprick blood was up to 7 times higher than in venous controls. Fluctuations in blood parameters with increasing volume of fingerprick blood are within instrument variability for volumes equal to or greater than 60 – 100 μL. These data suggest caution when using measurements from a single drop of blood.

Anemia is most common in regions of the world least able to afford effective diagnostics, and affects more than 60% of children under five years old in much of sub-Saharan Africa. Current methods to measure hemoglobin suffer from a high per-test cost or low accuracy. This thesis describes a method to measure hemoglobin concentration by performing spectroscopy of blood spotted on paper using a custom, portable reader. I assess the accuracy using chromatography paper in a U.S. laboratory and in a Malawi hospital. I assess the accuracy using nitrocellulose paper in the laboratory and in oncology patients at Lyndon B. Johnson hospital in Houston, TX, with 89% of samples within ±1 g/dL of a
reference standard. This method for measuring hemoglobin concentration costs <$0.01 per test.

Sickle cell disease is caused by a mutation in the hemoglobin molecule and leads to severe anemia, pain, and decreased immune function. It is estimated that 75.5% of babies with sickle cell disease are born in sub-Saharan Africa, where there are few resources to diagnose and treat the disease. Additionally, high-resource settings need a rapid diagnostic capable of identifying patients with sickle cell disease upon presentation to the emergency room. This thesis describes a new competitive lateral flow test capable of distinguishing sickle cell disease from sickle cell trait and normal blood using <10 μL of blood from a fingerprick with 98% accuracy (90% sensitivity and 100% specificity for identifying sickle cell disease).
Acknowledgments

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Worthy is the Lamb who was slain:
by his blood he ransomed the people for God.

- Revelation 5
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# Nomenclature

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<tr>
<td>POC</td>
<td>Point of care</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>WBC</td>
<td>White blood cell</td>
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<td>HbA</td>
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<tr>
<td>SCT</td>
<td>Sickle cell trait</td>
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Chapter 1

Introduction

1.1. Objectives and Specific Aims

The objective of this research is to develop separate devices to measure hemoglobin concentration and to determine sickle cell status. The specific aims are as follows:

Specific Aim 1: Design and build a low-cost, point-of-care method for measuring hemoglobin concentration.

Specific Aim 2: Evaluate the accuracy of the hemoglobin method in the field.

Specific Aim 3: Design, build, and test a low-cost, point-of-care method for determining sickle cell status.

1.2. Overview

This dissertation is organized as follows:
Chapter 2 provides background on anemia and sickle cell disease, current methods of diagnosis, and opportunities for improvement.

Chapter 3 describes work quantifying the drop-to-drop variation of hemoglobin, white blood cells, and platelets in successive drops of fingerprick blood. Though this work was completed partway through my dissertation research, its conclusions are important for the study designs in chapters 5 and 6. Chapter 3 was published previously in the American Journal of Clinical Pathology1.

Chapter 4 describes a spectroscopic method for measuring the hemoglobin concentration of blood spotted on chromatography paper. This chapter introduces the method and a version of a point-of-care reader and evaluates its performance on patient samples in the laboratory. Chapter 4 was published previously in Lab on a Chip2.

Chapter 5 presents improvements to the hemoglobin reader and evaluates its performance using patient samples at the point of care in the Queen Elizabeth Central Hospital in Blantyre, Malawi. Chapter 5 was previously presented as a contributed paper at the IEEE EMBS Special Topic Conference on Healthcare Innovation & Point-of-Care Technologies3.

Chapter 6 notes the improvements in hemoglobin concentration accuracy obtained when using nitrocellulose paper and covering the sample with Scotch tape as compared to uncovered chromatography paper. Further improvements to the reader are discussed, and the system accuracy is evaluated using patient samples obtained at the point-of-care at the Lyndon B. Johnson hospital in Houston, Texas.
Chapter 7 describes the development of a lateral flow test for distinguishing between unaffected patients, patients with sickle cell disease, and patients with sickle cell trait. The device is evaluated using blinded patient samples in the laboratory.

Chapter 8 provides a summary of the research presented in this thesis.
Chapter 2

Background

2.1. Hemoglobin Concentration

2.1.1. Definition and Need

Anemia is an insufficient concentration of the oxygen-carrying protein hemoglobin in the blood. Anemia affects a quarter of the world’s population (1.62 billion people), and can be caused by iron deficiency, malnutrition, blood loss, parasites (e.g. malaria, hookworm), and acute and chronic infections (e.g. tuberculosis, HIV), among others. Normal hemoglobin levels vary from lab to lab and can be affected by factors like gender, age, pregnancy status and trimester, altitude, and others, but the cutoffs below which people are considered anemic are generally 13 g/dL for men, 12 g/dL for non-pregnant women, and 11 g/dL for pregnant women and children under five years old.

Pregnant women and children under five have the highest prevalence of anemia due to increased iron requirements during periods of rapid growth. Globally, 41.8% of pregnant
women and 47.4% of preschool-age children suffer anemia (defined as <11 g/dL for these groups). The burden is even greater in low-resource areas such as Africa (57.1% of pregnant women and 67.7% of preschool-age children) and South-East Asia (48.2% of pregnant women and 65.5% of preschool-age children). Anemia can cause delayed mental and physical development, fatigue and decreased work productivity, and increased risk of mortality, especially during childbirth.

Anemia is diagnosed by measuring the concentration of hemoglobin in the blood. Once the condition is diagnosed, the underlying cause can be determined and treated with, for example, iron supplements, anti-malarial drugs, or blood transfusions. Hemoglobin concentration assessment is the most commonly performed laboratory test worldwide and is necessary for all healthcare systems. Performing this assessment at the point of care (POC) enables clinicians to make rapid decisions about the patient’s treatment.

2.1.2. Commercially Available Methods

Several methods exist to determine hemoglobin concentration. The gold standard method uses spectroscopy to measure hemoglobin after it has been converted to cyanmethemoglobin, a stable variant. Potassium ferricyanide \( (K_3Fe(CN)_6) \) converts hemoglobin to methemoglobin, and potassium cyanide \( (KCN) \) converts methemoglobin to cyanmethemoglobin. The absorbance of cyanmethemoglobin at 540 nm is directly proportional to the hemoglobin concentration. This method can be adversely affected by turbidity caused by lipidemia, high white cell count, or hemoglobin variants that make red blood cell (RBC) lysis difficult. This assessment method may be performed on an independent spectrometer or integrated as a separate channel in an automated cell counter. Additionally, the RBC count and hemoglobin concentration are linearly related if the RBCs
are normal\textsuperscript{9}. If the RBC count is available from an automated cell counter or other source, the hemoglobin concentration may be estimated as $[\text{Hb}] = 3 \times \text{RBC count}$. The cyanmethemoglobin method is highly accurate and used in many labs in the developed world, but it is impractical for the much of the developing world. The machines are bulky and expensive, and they require regular maintenance and reagents that rarely have a reliable supply chain in the developing world. The large size of the machine also precludes its use at the POC in developed or developing regions.

Many groups have sought to solve the shortcomings of the gold standard hemoglobin assessment method to create an appropriate point-of-care test. Two of the most well-known are the WHO Haemoglobin Colour Scale and the HemoCue.

The WHO Haemoglobin Colour Scale method requires blood from a fingerprick to be spotted onto filter paper. The color of this blood spot is compared to a standard color scale corresponding to hemoglobin concentrations from 4 g/dL to 14 g/dL in steps of 2 g/dL\textsuperscript{7}. This method meets many requirements for an appropriate POC test in low-resource settings: it is inexpensive, fast, requires no electricity and minimal training, and is easy to use at the POC. However, in ideal conditions the method is only accurate to within 2 g/dL, and with poor training or inadequate lighting, the accuracy can drop further. Medina Lara et al. found that 16\% of color scale tests deviated from the reference method by more than 2 g/dL\textsuperscript{8}, and Ingram et al. had 11.4\% differ by at least 2 g/dL when participants determined hemoglobin concentration without supervision\textsuperscript{7}. By comparison, clinicians in Malawi have requested accuracy to within $\pm 1$ g/dL for use in their hospital, and CLIA (Clinical Laboratory Improvement Amendments) standards for use in the U.S. require a device to be within $\pm 7\%$ of the standard.
The HemoCue 201+ (HemoCue AB, Ängelholm, Sweden) system draws blood into a plastic cuvette and makes spectroscopic measurements to return a hemoglobin concentration. The cuvette contains reagents to lyse the RBCs and convert the hemoglobin to azide methemoglobin. Absorbance measurements are made at 570 nm and 880 nm. Gomez-Simon et al. determined HemoCue to have a bias of ~0.8 g/dL (95% agreement limits -0.68 g/dL to 2.25 g/dL). Medina Lara et al. assessed the HemoCue, Haemoglobin Colour Scale, and other methods including the Jenway colorimeter, WBC colorimeter, DHT meter, and Laviband methods for use in determining hemoglobin concentration in Malawi. They considered accuracy and clinical usefulness, user friendliness, learning and usage time, and economic costs, and determined that HemoCue was the most appropriate method and the recommended standard-of-care in all Malawian district hospitals and urban health centers. However, the cost (approximately $1.00 in Malawi) of each single-use, plastic cuvette for HemoCue prevents widespread utilization of this accurate and easy-to-use hemoglobin concentration test at the community level, especially in low-resource rural areas where anemia prevalence is highest. This $1 to diagnose anemia represents a significant fraction of the $26 spent per capita on healthcare in 2013 in Malawi (according to the World Bank). Though the HemoCue device cost is high (approximately $450 in the US as of January 2016), it is often the recurring, per-test costs that serve as a barrier to continued use in low resource settings.

Thus, there is a need for an affordable, easy-to-use, accurate hemoglobin measurement system appropriate for the point of care. This thesis presents a method for measuring hemoglobin concentration at the point of care that addresses the problems of portability, ease of use, cost, and accuracy.
2.2. Sickle Cell Disease

2.2.1. Motivation and Need

A recessively inherited mutation of the hemoglobin gene causes sickle cell disease, which is clinically characterized by severe anemia, pain, and decreased immune function, among other symptoms\textsuperscript{10}. The mutated hemoglobin is known as hemoglobin S (or HbS), and normal hemoglobin is denoted hemoglobin A (or HbA). When deoxygenated, HbS changes conformation to expose a hydrophobic site, which binds to a complementary site on another HbS molecule. Long chains of HbS molecules form inside a red blood cell (RBC) and deform it into the characteristic sickled shape. These cells can occlude vessels, causing pain, are destroyed more rapidly than normal RBCs, causing anemia, and block vessels in the spleen designed to filter RBCs and bacteria, leading to decreased immune function. Homozygotes for the sickle cell gene mutation (genotype SS) are affected by the disease. Heterozygotes (genotype AS) have sickle cell trait and suffer few symptoms of the disease, though they can pass on the gene to their children.

Global distribution of the sickle cell mutation (\textbf{Figure 2-1}) follows the historic distribution of malaria, as heterozygosity for sickle cell offers some protection against dying from malaria. Piel et al. estimated that 312,302 babies were born with sickle cell disease in 2010 and a further 5,476,407 were born with sickle cell trait\textsuperscript{11}. An estimated 75.5\% of babies with sickle cell disease are born in sub-Saharan Africa, where there are few resources to diagnose and treat the disease\textsuperscript{11}. As treatment for sickle cell disease continues to improve and under-five mortality is reduced in general, the number of people in low-resource settings requiring management of sickle cell disease is expected only to grow\textsuperscript{11}. Despite recent advances, morbidity and mortality from sickle cell disease are still high. Lifespans for people
with sickle cell disease in the U.S. are an average 3 decades shorter than the general population.\textsuperscript{12}

![Figure 2-1](image)

**Figure 2-1.** Global distribution of the HbS allele. Figure adapted from Piel et al.\textsuperscript{11} Reprinted with permission from Elsevier (The Lancet, 2013, 381, 142-151).

In high-resource settings, therapies are available to reduce painful sickle cell crises, increase hemoglobin concentration, and prevent infection. Blood transfusions from unaffected donors and hydroxyurea therapy, which increases the production of non-sickling fetal hemoglobin (HbF), serve to dilute the number of sickled RBCs and increase hemoglobin concentration.\textsuperscript{12} Early in life, sickled red blood cells cause vasco-occlusion in the spleen, eventually leading to functional hyposplenism or asplenism. Without the filtering effects of the spleen, children are vulnerable to encapsulated organisms, especially *S. pneumoniae*.\textsuperscript{13} Daily oral penicillin until the age of five years is used to reduce mortality from these infections.\textsuperscript{12} These treatments, combined with universal newborn screening to identify patients quickly, contribute to 90-99% of children with sickle cell disease surviving to the age of five years in developed countries.\textsuperscript{14} However, only an estimated 20-50% of affected
children in Africa survive to the age of five\textsuperscript{10}. One of the barriers to treatment in low-resource settings is lack of robust newborn screening programs to identify patients in need of treatment\textsuperscript{14}, and there is a need for a low-cost, rapid, easy-to-use tool for screening purposes.

For patients in the developed world suffering from a painful vaso-occlusive crisis, a hallmark of sickle cell disease, the current recommendation is to rapidly treat with opioid pain relievers\textsuperscript{12}. However, when presenting at the emergency room, these patients can easily be mistaken as drug-seeking or drug-addicted patients. A rapid diagnostic appropriate for use in an emergency room would help distinguish sickle cell patients in true need from drug seekers or drug-addicted patients. Additionally, an autopsy study of causes of death in U.S. sickle cell patients suggests that a large percentage of deaths (27.7\%) occurred in the first 24 hours after presentation of the patient to the hospital, so rapid treatment during these crises is essential\textsuperscript{15}. Thus, there is a need in the developed and developing worlds for a tool to rapidly identify patients with sickle cell disease for applications in newborn screening programs or emergency care.

2.2.2. Commercially Available Diagnostics and Screening Tools

Diagnostic tests have a high sensitivity and specificity for identifying sickle cell and are typically used when a patient presents with symptoms consistent with sickle cell or to confirm the result of a screening test. Screening tests are used to identify affected patients before they present with symptoms and may have a lower sensitivity or specificity than diagnostic tests, which are used to confirm positive results. In high prevalence areas, an increased specificity is required to correctly identify patients without the disease. As
prevalence decreases, an increased sensitivity is required to maintain a given positive predictive value, identifying the small number of affected patients.

Isoelectric focusing (IEF) and high performance liquid chromatography (HPLC) are considered gold standards for sickle cell diagnosis\(^\text{16}\). In IEF, proteins from lysed blood are run on an agarose gel with a pH gradient. With the application of an electric field, the proteins move through the agarose to a location with pH matching their isoelectric point\(^\text{17}\). IEF separates hemoglobins A, S, and F, as well as other hemoglobin mutations. HPLC may be used to separate hemoglobin mutations based on interaction time with a column, and it readily separates hemoglobins A, S, F, and other mutations\(^\text{17}\). Both methods may be used to quantify the relative amounts of each type of hemoglobin in the sample. While these diagnostic methods are sensitive and specific, both require expensive equipment and extensive training and are thus not appropriate for point-of-care use, especially in low resource settings.

Several tests have been developed to screen for sickle cell at the point of care. Tests like SickleDex (Streck, Omaha, NE) exploit solubility differences in the HbA and HbS molecules. Whole blood is added to a liquid containing reagents to lyse the red blood cells (saponin), deoxygenate the hemoglobin (sodium hydrosulfite), and induce precipitation in the HbS molecule (high concentration of salt)\(^\text{17}\). If present, the HbS precipitates and makes the solution turbid, while solutions lacking HbS remain clear. SickleDex is $3.40 per test when purchased from Ficher Scientific (as of January 2016). SickleDex is simple to use and sensitive for the presence of HbS, but it is not specific. False positives can be caused by sickle cell trait, severe anemia, blood from neonates with high concentrations of HbF, and other factors\(^\text{17}\). Sickle cell trait, blood from neonates, and anemia can all be expected in a
newborn screening setting in a region with high prevalence, so it is important that a test give accurate results under these conditions.

Lateral flow or strip tests have the potential to meet the World Health Organization’s (WHO) ASSURED criteria for diagnostic tests (accurate, sensitive, specific, user-friendly, rapid, robust, equipment-free, and deliverable) for sickle cell screening and diagnosis. During the writing of this thesis, data from Sickle SCAN (Biomedomics, Research Triangle Park, NC) were published\textsuperscript{18,19}. Sickle SCAN is a novel lateral flow test for detecting combinations of HbA, S, and C. To operate the test, 5 μL of blood is collected from the patient and then mixed with a lysis and diluent buffer. Several drops of this mixture are applied to the lateral flow strip, and blue lines form via a sandwich assay to indicate the types of hemoglobin present in the sample (Figure 2-2).

![Image of Sickle SCAN test results](image.png)

**Figure 2-2.** Figure adapted Kanter et al.\textsuperscript{18} showing possible results using Sickle SCAN.

McGann et al. report a high sensitivity (98.3-100%) and specificity (92.5-100%) for the device, however, it requires a precise measurement of blood volume, and the user must
dilute the blood before adding it to the device. A lateral flow strip that could accept
unmeasured blood directly from a fingerprick would reduce cost and substantially reduce the
requirements on the user.

Thus, there is a need for a low cost, point-of-care test that can distinguish among
HbSS, HbAS, and HbAA, is sensitive and specific, and is easy to use. This thesis presents a
new lateral flow strip to accomplish these goals.
Chapter 3

Drop-to-Drop Variation in the Cellular Components of Fingerprick Blood: Implications for Point-of-Care Diagnostics Development*

* The contents of this chapter have been published in the following journal article: Bond, MM and Richards-Kortum, RR. Drop-to-Drop Variation in the Cellular Components of Fingerprick Blood: Implications for Point-of-Care Diagnostic Development. *Am J Clin Pathol.* 2015; 144(6):885-94, by permission of the American Society for Clinical Pathology and the Academy of Clinical Laboratory Physicians and Scientists.
3.1. Abstract

3.1.1. Objectives

Blood obtained via fingerprick is commonly used in point-of-care assays, but few studies have assessed variability in parameters obtained from successive drops of fingerprick blood, which may cause problems for clinical decision making and for assessing accuracy of point-of-care tests.

3.1.2. Methods

We used a hematology analyzer to analyze the hemoglobin concentration, total white blood cell (WBC) count, 3-part WBC differential, and platelet count in 6 successive drops of blood collected from one fingerprick of 11 donors, and we used a hemoglobinometer to measure the hemoglobin concentration of 10 drops of fingerprick blood from 7 donors.

3.1.3. Results

The average percent coefficient of variation (CV) for successive drops of fingerprick blood was higher by up to 3.4 times for hemoglobin, 5.7 times for WBC count, 3.0 times for lymphocyte count, 7.7 times for granulocyte count, and 4 times for platelets than in venous controls measured using a hematology analyzer. The average percent CV for fingerprick blood was up to 5 times higher for hemoglobin than venous blood measured using a point-of-care hemoglobinometer. Fluctuations in blood parameters with increasing volume of fingerprick blood are within instrument variability for volumes equal to or greater than 60 – 100 µL.
3.1.4. Conclusions

These data suggest caution when using measurements from a single drop of fingerprick blood.

3.2. Introduction

Blood obtained via fingerprick is commonly used in point-of-care assays because fingerpricks are less invasive than venipuncture, they require less clinical training than venipuncture, and their small blood volume is sufficient for point-of-care tests. Accuracy in these tests is important for diagnosing anemia or infection and managing HIV, sickle-cell anemia, malaria, and other diseases, especially in low-resource settings where performing venipuncture and using a hematology analyzer is not feasible. Many researchers have examined differences in blood parameters for fingerprick (or fingerstick) and venous blood.20–24 A few researchers have examined variations in blood parameters for different fingerprick protocols by comparing results for fingerpricks performed on both hands, on different days, using different devices, or by comparing several drops of blood from a fingerprick.25–29 However, few studies have analyzed the variation in blood parameters between the successive drops of blood obtained from one fingerprick. Because of the growing number of clinically important tests performed using fingerprick blood, especially in low-resource settings, it is important to understand how variations in fingerprick blood collection protocols can affect point-of-care test accuracy and the potential variability introduced when two point-of-care blood tests are performed using fingerprick blood from the same patient.
The goal of this pilot study was to determine the drop-to-drop variability in blood parameters obtained from fingerprick blood. We also aimed to determine the minimum volume of blood needed to reduce variability to acceptable levels for clinical decision making, such as determining if a patient is anemic. To answer these questions, we analyzed the hemoglobin concentration, total white blood cell (WBC) count, 3-part WBC differential (lymphocytes, monocytes, and granulocytes), and platelet count in 6 successive drops (20 μL each) of blood collected from one fingerprick using a hematology analyzer. Venous blood was drawn for comparison. This study also assessed the variability of the hemoglobin concentration of 10 successive drops (10 μL each) of fingerprick blood when measured using a point-of-care hemoglobinometer.

### 3.3. Materials and Methods

We assessed drop-to-drop variation of blood parameters using a laboratory-grade hematology analyzer (Ac•T diff2, Beckman-Coulter, Brea, CA). The hematology analyzer reported hemoglobin concentration, total WBC count, 3-part WBC differential, and platelet count. To validate the use of small sample volumes on this device, we first measured successive drops of venous blood from a volunteer donor. Then, we measured successive drops of blood from fingerpricks of volunteer donors. In both cases, blood samples were collected with separate 20 μL MicroSafe capillary tubes (SafeTec, Ivyland, PA), dispensed into tubes with pre-measured diluent, and analyzed in pre-dilute mode.

We also assessed drop-to-drop variation in hemoglobin concentration on a device designed to be used at the point-of-care (HemoCue 201+, HemoCue AB, Ängelholm, Sweden). We first measured drops of venous blood to validate the repeatability of the device.
itself; then, we measured successive drops of blood from fingerpricks of donors. For measurement on this device, 10 μL blood samples were collected directly into a HemoCue disposable cuvette.

3.3.1. **Small volumes of venous blood measured using a hematology analyzer**

To validate the method of measuring small volumes of blood using the hematology analyzer, venous blood was analyzed using the same procedure as fingerprick blood. Normal volunteer blood was used undiluted and diluted with human plasma to simulate various levels of anemia and leukopenia. Blood was obtained from volunteers (healthy, non-pregnant adults weighing at least 110 pounds) who gave written informed consent. Plasma for diluting blood was purchased from the Gulf Coast Regional Blood Bank (Houston, TX). Both protocols were reviewed and approved by the Institutional Review Board at Rice University.

Venous blood samples were well-mixed, and 6 drops of approximately 25 μL were pipetted onto Parafilm (Bemis, Oshkosh, WI). Each drop was drawn into a 20 μL MicroSafe capillary tube. An air vent in the wall of the plastic tube regulated the amount of blood collected (within 0 to +10% of the stated volume), and the integrated bulb facilitated dispensing of the blood. To measure a complete blood count using such a small volume of blood accurately on the Act-Diff 2 hematology analyzer, the pre-dilute function was used. (In this mode, the Act-Diff 2 pre-dispenses 1580 μL of diluent into a tube. The user adds 20 μL of blood, mixes the sample well, and presents it to the analyzer, which performs the necessary calculations to account for the dilution. This procedure is specified in the operator’s manual.) The drops of venous blood collected in the MicroSafe tubes were dispensed using the integrated bulb into the tubes with pre-measured diluent, and the
solution was mixed well. These tubes were analyzed on the Ac•T diff2 for hemoglobin concentration, WBC count, 3-part WBC differential, and platelet count.

3.3.2. Drop to drop variation in fingerprick blood parameters measured using a hematology analyzer

Healthy volunteers had 3 – 9 mL of blood drawn into appropriately sized K2 EDTA vacutainer (BD, Franklin Lakes, NJ) tubes by venipuncture. Then, the side of the third or fourth finger was warmed, cleansed with an alcohol wipe, and pricked with a BD Contact-Activated Lancet (High Flow, 1.5 mm blade, 2.0 mm depth, product number 366594; BD, Franklin Lakes, NJ), except for donor A, who was pricked with a Unistik 3 Dual lancet (18G needle, 1.8mm depth, product number AT 1062; Owen Mumford, Oxford, UK). The initial drop of blood to form at the puncture site was wiped away with sterile gauze in accordance with standard procedures. This drop was wiped away because of the possible contamination of the drop of blood with alcohol, cell debris, and tissue fluids. The next 6 successive drops to form at the site were collected with separate 20 μL MicroSafe capillary tubes. If blood flow began to slow, the puncture site was wiped firmly with sterile gauze to remove the platelet plug and encourage further blood flow. The puncture sites were not “milked” to encourage blood flow as this action can lead to erroneous results, such as a falsely low hemoglobin concentration. Fourteen donors were recruited; data from 3 donors were rejected from analysis because the fingerpricks required milking to reach 6 drops or had clots in the first 6 drops.

Using the integrated bulb, the drops of blood collected in the MicroSafe tubes were dispensed into the tubes with pre-measured diluent, and the tubes were mixed well. Each drop was analyzed on the Ac•T diff2 analyzer, followed by analysis of the donor's venous
blood in whole blood mode. A single investigator (MMB) trained and certified in performing
fingerpricks collected all blood and performed all experiments, and all blood from a single
donor was measured on the same day as soon as possible after collection.

3.3.3. Drop-to-drop variation in fingerprick blood parameters measuring using a
point-of-care device

We also assessed the variation of hemoglobin concentration in successive drops of
fingerprick blood using a HemoCue 201+. To establish the degree of variability caused by
the device itself, venous blood samples were well-mixed, and 10 drops of approximately 15
µL were pipetted onto Parafilm (Bemis, Oshkosh, WI), drawn into the HemoCue 201+
cuvettes (10 µL volume) by capillary action, and analyzed for hemoglobin concentration.
One blood sample was used undiluted and diluted with varying amounts of human plasma to
simulate degrees of anemia.

To assess drop-to-drop variability in fingerprick blood, volunteers were recruited,
and their third or fourth fingers were pricked using the same procedure as above, but with a
Unistik 3 Dual lancet (18G needle, 1.8mm depth, product number AT 1062; Owen
Mumford, Oxford, UK). The first drop was wiped away, and 10 successive drops
(approximately 10 µL each) were collected directly into the HemoCue 201+ cuvettes.
Samples were analyzed in order of collection as soon as the last drop was collected (within
the recommended 10 minutes after collection). None of the 7 donors recruited required
milking to reach 10 drops or had clots in the first 10 drops. Venous blood was not drawn
from these donors. A single investigator (MMB) trained and certified in performing
fingerpricks collected all blood and performed all experiments.
3.4. Results

3.4.1. Drop-to-drop variation measured using a hematology analyzer

Figure 3-1. Hemoglobin concentration measured using hematology analyzer.
(a) Low variability for venous blood measured using hematology analyzer. Normalized hemoglobin concentration of 6 drops (20 μL each) of venous samples at 3 hemoglobin levels. i = drop number. (b) High variability for drops of fingerprick blood measured using hematology analyzer. Normalized hemoglobin concentration of 6 successive drops (20 μL each) of fingerprick blood from 11 donors. (c) Variability for fingerpricks decreases when averaging multiple drops. Normalized running average of hemoglobin concentration of the drops in (b). (d) Comparison of fingerprick hemoglobin concentration to venous hemoglobin concentration. Difference between running average of hemoglobin concentration of the drops in (b) and the venous hemoglobin concentration of the same donor.
We first used a hematology analyzer to assess the drop-to-drop variation in measuring small volumes (20 μL) of venous blood. Theoretically, the drop-to-drop variation of drops taken from a well-mixed sample is zero. The variation of hemoglobin concentration in 6 drops of venous blood is shown graphically in Figure 3-1a and numerically in Table 3-1. The variation was slightly higher in samples with a higher concentration of hemoglobin but in all cases compared favorably with the reported accuracy of the hematology analyzer (<2% coefficient of variation [CV] for 31 samples, according to the instrument manual).

We then measured the variability in successive drops (20 μL) of fingerprick blood. The hemoglobin concentration of 6 successive drops of fingerprick blood from 11 donors is shown in Figure 3-1b. The hemoglobin concentration of the last drop of blood has been

<table>
<thead>
<tr>
<th></th>
<th>Successive 20 μL drops of venous blood</th>
<th>Successive 20 μL drops of fingerprick blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td>Average</td>
<td>14.8</td>
<td>9.5</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.28</td>
<td>0.15</td>
</tr>
<tr>
<td>%CV</td>
<td>1.9%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Range</td>
<td>0.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>
subtracted from that of each drop to better visualize drop-to-drop changes independent of the baseline hemoglobin concentrations of the donors. Table 3-1 summarizes these data numerically; the standard deviation, percent CV, and range (maximum – minimum value) were determined for each donor and then averaged for all donors. (Data are broken down by donor in Table A 1, Appendix.) The average percent CV for successive drops of fingerprick blood was between 2.3 and 3.4 times greater than that measured for small volumes of venous blood. Indeed, taking multiple drops of blood from one fingerprick can result in hemoglobin concentrations that differ by more than 1.6 g/dL. Note that there was no generalizable trend of the change in hemoglobin concentration as more drops are collected. For example, in Figure 3-1b, Donor B shows a decrease of > 2 g/dL from baseline in drop 3, but returns to baseline in drop 5. Not all donors showed large changes across multiple drops: Donor F deviates < 0.5 g/dL from baseline across all 6 drops. Data from donors whose blood was not freely flowing for 6 drops were rejected from analysis, so these variations from drop to drop are not due to “milking” the finger.

Figure 3-1c shows the running average of the hemoglobin concentration of all previous drops of blood, shown individually in Figure 3-1b. The average of all 6 drops for each donor has been subtracted from each point to remove the effects of the donor’s baseline hemoglobin concentration. For all 11 donors, fluctuations in hemoglobin concentration with increasing sample volume of fingerprick blood are within instrument variability (0.5 g/dL, calculated by averaging the ranges of the 3 venous samples measured using the hematology analyzer) for volumes equal to or greater than 60 μL (3 drops).

Figure 3-1d shows the difference between the running average of fingerprick hemoglobin concentration and the venous hemoglobin concentration for the same donor.
For 9 of the 11 donors, the running average of fingerprick hemoglobin concentration was within $\pm 1.1 \text{ g/dL}$ of their venous hemoglobin concentration for all cumulative volumes. Donors B and C showed a greater deviation between fingerprick and venous hemoglobin concentration despite careful attention to proper blood collection procedures, including not leaving the tourniquet on for too long during venipuncture and not milking the finger during the fingerprick.
Figure 3-2. WBC concentration measured using hematology analyzer. (a) Low variability for venous blood measured using hematology analyzer. Normalized WBC concentration of 6 drops (20 μL each) of venous samples at three WBC levels. \( i \) = drop number. (b) High variability for drops of fingerprick blood measured using hematology analyzer. Normalized WBC concentration of 6 successive drops (20 μL each) of fingerprick blood from 11 donors. (c) Variability for fingerpricks decreases when averaging multiple drops. Normalized running average of WBC concentration of the drops in (b). (d) Comparison of fingerprick WBC concentration to venous WBC concentration. Difference between running average of WBC concentration of the drops in (b) and the venous WBC concentration of the same donor.
Table 3-2. WBC concentration and 3-part differential measured using hematology analyzer. The left side of the table shows the average, standard deviation, percent CV, and range (maximum – minimum value) of the samples depicted in Figure 3-2a (venous blood). The right side of the table shows statistics for samples shown in Figure 3-2b (fingerprick blood). For the fingerpricks, measures were calculated for 6 drops from one fingerprick of each donor, then averaged for all donors.

<table>
<thead>
<tr>
<th></th>
<th>Successive 20 μL drops of venous blood</th>
<th>Successive 20 μL drops of fingerprick blood</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WBC Concentration (x10^6 cells/μL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>1.5%</td>
<td>2.1%</td>
</tr>
<tr>
<td>St Dev</td>
<td>0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

| **Lymphocyte Count (x10^6 cells/μL)** |                                        |                                              |
| Average                | Sample 1 | Sample 2 | Sample 3 | St Dev | %CV | Range | Average St Dev | %CV | Range |
|                        | 1.7      | 1.5      | 1.2      | 0.04   | 2.4% | 0.1    | 0.18            | 7.2%| 0.5   |

| **Granulocyte Count (x10^6 cells/μL)** |                                        |                                              |
| Average                | Sample 1 | Sample 2 | Sample 3 | St Dev | %CV | Range | Average St Dev | %CV | Range |
|                        | 4.8      | 4.8      | 3.4      | 0.06   | 1.3% | 0.2    | 0.42            | 10% | 1.1   |

| **Monocyte Count (x10^6 cells/μL)** |                                        |                                              |
| Average                | Sample 1 | Sample 2 | Sample 3 | St Dev | %CV | Range | Average St Dev | %CV | Range |
|                        | 0.3      | 0.2      | 0.1      | 0.05   | 21.9%| 0.1    | 0.08            | 30% | 0.2   |
Figure 3-2 and Table 3-2 show the results of the same analyses performed on WBC measures (WBC count and absolute number of lymphocytes, granulocytes, and monocytes). (Data are broken down by donor in Table A 1, Appendix.) Figure 3-2a shows the WBC concentration of multiple drops of venous blood at three levels of WBC concentration with the concentration of the final drop subtracted from each drop to normalize each sample. Figure 3-2b shows the WBC concentration of successive drops of fingerprick blood from 11 donors with the concentration of the final drop subtracted from each drop. Figure 3-2c shows the running average of WBC concentration for the drops in 2b with the average of all 6 drops subtracted from each point to normalize each sample. For all 11 donors, fluctuations in WBC concentration with increasing sample volume of fingerprick blood are within instrument variability (0.3 x 10^6 cells/μL, calculated by averaging the ranges of the 3 venous samples measured using the hematology analyzer) for volumes equal to or greater than 100 μL (5 drops). For 10 of the 11 donors, fluctuations are within instrument variability for volumes equal to or greater than 80 μL (4 drops). Figure 3-2d shows the running average of fingerprick drops with the venous WBC concentration subtracted from each point.

The standard deviation, percent CV, and range of data taken from 6 drops of venous blood were low; the percent coefficient variation for WBC count was within the reported accuracy of the device, and the standard deviations for monocytes and granulocytes were within the reported accuracy of the device. Our results showed a slightly higher standard deviation for lymphocytes than the reported accuracy of the device; this deviation may have been due to our method of dilution. Monocytes had a high percent CV compared to the other WBC types due to their low absolute count in these samples from healthy volunteers.
In contrast to the results for venous blood, all measures of WBC variability were higher for successive drops of fingerprick blood, except for the monocyte percent CV, which was high and comparable to that measured in venous controls. The average percent CV for successive drops of fingerprick blood was 3.9 to 5.7 times higher for WBC count, 1.4 to 3.0 times higher for lymphocyte count, and 3.2 to 7.7 times higher for granulocyte count than in venous controls.

![Figure 3-3](image)

Figure 3-3. Platelet (Plt) concentration measured using hematology analyzer. (a) Low variability for venous blood measured using hematology analyzer. Normalized platelet concentration of 6 drops (20 μL each) of venous samples at three platelet concentrations. i = drop number. (b) High variability for drops of fingerprick blood measured using hematology analyzer. Normalized platelet concentration of 6 successive drops (20 μL each) of fingerprick blood from 11 donors. (c) Variability for fingerpricks decreases when averaging multiple drops. Normalized running average of platelet concentration of the drops in (b). (d) Comparison of fingerprick platelet concentration to venous platelet concentration. Difference between running average of platelet concentration of the drops in (b) and the venous platelet concentration of the same donor.
Table 3-3. Platelet concentration measured using hematology analyzer. The left side of the table shows the average, standard deviation, percent CV, and range (maximum – minimum platelet value) of the platelet concentration of samples depicted in Figure 3-3a (venous blood) in g/dL. The right side of the table shows statistics for the platelet concentration of samples depicted in Figure 3-3b (fingerprick blood). For the fingerpricks, measures were calculated for 6 drops collected from one fingerprick of each donor, then averaged for all donors.

<table>
<thead>
<tr>
<th></th>
<th>Successive 20 μL drops of venous blood</th>
<th>Successive 20 μL drops of fingerprick blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td>Average</td>
<td>316</td>
<td>238</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>14.6</td>
<td>11.4</td>
</tr>
<tr>
<td>%CV</td>
<td>4.6%</td>
<td>4.8%</td>
</tr>
<tr>
<td>Range</td>
<td>42</td>
<td>32</td>
</tr>
</tbody>
</table>

Figure 3-3 and Table 3-3 summarize the results of the same analyses performed on platelet concentration. (Data are broken down by donor in Table A 1, Appendix.) The variation of venous samples was within the reported accuracy of the hematology analyzer (<7% coefficient of variation [CV] for 31 samples, according to the instrument manual). The average percent CV for successive drops of fingerprick blood was 4 times greater than that measured for small volumes of venous blood. Figure 3-3a shows the platelet concentration of multiple drops of venous blood at three levels of platelet concentration with the concentration of the final drop subtracted from each drop to normalize each sample. Figure 3-3b shows the platelet concentration of successive drops of fingerprick blood from 11 donors with the concentration of the final drop subtracted from each drop. Figure 3-3c
shows the running average of platelet concentration for the drops in 3b with the average of all 6 drops subtracted from each point to normalize each sample.

Only 5 of the 11 donors had fluctuations in platelet concentration with increasing sample volume of fingerprick blood within instrument variability (33 platelets/μL, calculated by averaging the ranges of the 3 venous samples measured using the hematology analyzer) for volumes of 120 μL (6 drops). Figure 3-3d shows the running average of fingerprick drops with the venous platelet concentration subtracted from each point.

In general, platelet concentrations decreased in each successive drop from the fingerpricks. Additionally, venous platelet concentrations were generally higher than platelet concentrations in fingerprick blood. These findings may reflect the consumption of platelets during the clotting process observed after a fingerprick.
3.4.2. Drop-to-drop variation on a point-of-care device

![Figure 3-4](image)

**Figure 3-4.** Hemoglobin concentration measured using point-of-care hemoglobinometer. (a) Low variability for venous blood measured using point-of-care device. Normalized hemoglobin concentration of 10 drops (10 μL each) of venous samples at three hemoglobin levels. (b) High variability for drops of fingerprick blood measured using point-of-care device. Normalized hemoglobin concentration of 10 successive drops (10 μL each) of fingerprick blood from 7 donors. (c) Variability for fingerpricks decreases when averaging multiple drops. Normalized running average of hemoglobin concentration of the drops in (b).
We also assessed the drop-to-drop variation of blood measured using a point-of-care hemoglobinometer, the HemoCue 201+. We first assessed the drop-to-drop variation in drops (10 μL) of venous blood. Theoretically, the drop-to-drop variation of drops taken from a well-mixed sample is zero. The variation of hemoglobin concentration in 10 drops of venous blood is shown numerically in Table 3-4. (Data are broken down by donor in Table A 2, Appendix.) The range of variability for venous blood was within the manufacturer’s reported accuracy for the HemoCue 201+. The average percent CV was 2.2 to 5 times higher when measuring fingerprick blood than venous blood.

Table 3-4. Hemoglobin concentration measured using point-of-care device. The left side of the table shows the average, standard deviation, percent CV, and range (maximum – minimum hemoglobin value) of the samples depicted in Figure 3-4a (venous blood) in g/dL. The right side of the table shows statistics for the samples depicted in Figure 3-4b (fingerprick blood). For the fingerpricks, measures were calculated for the collection of drops from one fingerprick of each donor, then averaged for all donors.

<table>
<thead>
<tr>
<th>Successive 10 μL drops of venous blood</th>
<th>Successive 10 μL drops of fingerprick blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td>Average</td>
<td>14.2</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.10</td>
</tr>
<tr>
<td>%CV</td>
<td>0.7 %</td>
</tr>
<tr>
<td>Range</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Figure 3-4a shows the hemoglobin concentration of multiple drops of venous blood at three hemoglobin concentrations, with the concentration of the final drop subtracted from each point. Figure 3-4b shows the hemoglobin concentration of 10 successive drops of fingerprick blood from 7 donors with the concentration of the final drop subtracted from each drop to better visualize the drop-to-drop changes regardless of baseline hemoglobin concentration.

Figure 3-4c shows the running average of hemoglobin concentration of the drops shown in Figure 3-4b with the average of all 10 drops subtracted from each point. For all donors, fluctuations in hemoglobin concentration with increasing sample volume of fingerprick blood are within instrument variability (0.3 g/dL, calculated by averaging the ranges of the 3 venous samples measured using the point-of-care device) for volumes greater than 90 μL (9 drops). When using the same target instrument variability as the hematology analyzer (0.5 g/dL), we find that for all donors, fluctuations in hemoglobin concentration with increasing sample volume of fingerprick blood are within 0.5 g/dL for volumes greater than 60 μL (6 drops).

3.5. Discussion

Using both a hematology analyzer and point-of-care hemoglobinometer, we found the variability of blood component measures to be greater for successive drops of fingerprick blood than for multiple drops of venous blood. Our measurements of average percent CV for hemoglobin concentration from fingerprick blood (4.4% for hematology analyzer and 3.5% for the point-of-care device) were comparable to literature reports of similar experiments. Yang et al. measured the hemoglobin concentration of three drops (20
µL each) from a fingerprick and found an average percent CV of 2.45 ± 1.32 %. Morris et al. found a CV of 6.3% when comparing hemoglobin concentration from fingerpricks on the right hand to those collected simultaneously on the left hand. Chen et al. found a CV of 8.0% when comparing hemoglobin concentration of the second drop to the third drop of a fingerprick.

Our measurements of venous platelet variation (4.6 – 4.8% CV) are similar to those measured by Yang et al. (4.50 ± 3.02 % CV). However, our measurement of platelet variation in fingerprick blood is markedly greater: 19% CV as compared to 6.47 ± 6.57 % CV. Our measurements are more comparable with those of Brecher et al., who found 11% CV in venous blood and 24% CV in fingerprick blood.

Here, similar degrees of variability were seen in the hemoglobin concentration of venous blood on both the hematology analyzer and point-of-care hemoglobinometer. The measurements taken on the Ac•T diff2 may have been slightly more variable due to the number of steps involved in measuring small amounts of blood on the analyzer. The HemoCue method is designed to measure small volumes of blood, so the workflow is simpler, which may contribute to reduced variability on well-mixed samples. We do not believe that the Microsafe capillary tubes were the source of the variability, since they were only used when measuring blood using the hematology analyzer, and the measures of hemoglobin variability were similar on both devices.

Similar degrees of variability were also seen in the hemoglobin concentration of successive drops of fingerprick blood on both the hematology analyzer and point-of-care hemoglobinometer. The literature reports conflicting results concerning the accuracy of the HemoCue 201+ and its predecessors. Studies that assess the device’s accuracy using venous
blood tend to show excellent agreement with laboratory hemoglobinometers; studies that assess the device’s accuracy in the field, using fingerprick blood, tend to report much poorer accuracy, and some of these studies recommend against using the HemoCue 201+ entirely due to its poor accuracy. The results presented in Figure 3-4 of this paper suggest that the cause of this discrepancy in reported results is the sample itself: when venous blood is used, the HemoCue gives accurate results; when fingerprick blood is used, the HemoCue is affected by the inherent variability of drops of fingerprick blood.

The fact that similar trends were observed for both hemoglobin concentration and WBC concentration measured using the hematology analyzer (e.g., donor B showed a decrease in drop #3 in both hemoglobin concentration [Figure 3-1b] and WBC concentration [Figure 3-2b]) suggests that the changes from drop to drop are due to a different ratio of cellular components to plasma. Because donors were only included in the analysis if all drops could be obtained without “milking” the finger, we do not believe this effect was induced by the person collecting the blood. Morris et al. believe the higher variability of capillary blood as compared to venous blood is due to the presence of extracellular fluid in capillary samples. In clinical practice, “milking” of the finger by insufficiently trained healthcare workers may result in even greater drop-to-drop variability than shown here.

Our data also suggest that collecting and analyzing more fingerprick blood does not necessarily bring the measured value closer to those of the donor’s venous blood (Figure 3-1d and Figure 3-2d). For example, donor B’s hemoglobin and WBC concentration were similar for venous blood and fingerprick in drop 1, but became less concordant with additional drops, while donor C’s fingerprick measures came closer to the venous measures
with additional drops. These data may represent true differences between fingerprick and venous blood, or they may be the result of errors in collection (such as leaving the tourniquet on for too long during a venous draw). Further research is needed to determine how common these patterns are.

For testing the accuracy of new devices using fingerprick blood, we recommend collecting multiple drops of fingerprick blood in an anticoagulant-coated tube (such as BD’s Microtainer Tubes), mixing the blood thoroughly with a pipette, and then apportioning the blood to each device under investigation. This method ensures that a test device and reference standard measure blood with the same concentration of components.

For clinical decision-making, we recommend using fingerprick blood to assess hemoglobin or WBC concentration only when the degree of variability is acceptable (the degree of acceptable variability will depend on the clinical condition being assessed - for example, a clinician may desire higher accuracy when assessing the need for a blood transfusion than when assessing iron-deficiency anemia). Other studies have examined more closely the bias of capillary blood when compared to venous blood, and Neufeld et al. even suggest a conversion factor. However, these averages do not account for the large variability of hemoglobin and WBC concentration from drop to drop of fingerprick blood. Our data suggest that the running average of hemoglobin and WBC concentration stops changing after averaging 80 μL of fingerprick blood. That is, collecting more than 80 μL provides little additional information. This volume is similar to the 4-9 drops (40-90 μL) recommended by Morris et al. It should be noted that this volume was derived by mathematically combining individual, separately tested drops of blood; further studies should verify that combining drops before analysis reduces variability to acceptable levels.
In recent years, a large number of devices using tiny volumes of blood have been developed. Our data suggest caution in using the results of these hemoglobin and WBC tests for clinical decision-making, such as determining anemia status. (Other studies need to be conducted to assess the drop-to-drop variability for other analytes.) The options for clinicians seem to be to (1) accept the inaccuracy of fingerprick blood on these devices as a tradeoff for easy blood collection; (2) collect, read, and average multiple fingerprick samples, gaining accuracy but sacrificing cost and time; or (3) collect and analyze venous blood.

3.6. Acknowledgments

We would like to thank our volunteer blood donors for making this study possible.
Chapter 4

Chromatography Paper as a Low Cost Medium for Accurate Spectrophotometric Assessment of Blood Hemoglobin Concentration†

4.1. Abstract

Anemia affects a quarter of the world’s population, and a lack of appropriate diagnostic tools often prevents treatment in low-resource settings. Though the HemoCue

† The contents of this chapter have been published in the following journal article: Bond, M, Elguea C, Yan JS, Pawłowski M, Williams J, Wahed A, Oden M, Tkaczyk TS, and Richards-Kortum, R. Chromatography paper as a low-cost medium for accurate spectrophotometric assessment of blood hemoglobin concentration. Lab Chip, 2013; 13(12):2381-8. Reproduced with permission from the Royal Society of Chemistry.
201+ is an appropriate device for diagnosing anemia in low-resource settings, the high cost of disposables ($0.99/test in Malawi) limits its availability. We investigated using spectrophotometric measurement of blood spotted on chromatography paper as a low-cost (<$0.01/test) alternative to HemoCue cuvettes. For this evaluation, donor blood was diluted with plasma to simulate anemia, a micropipette spotted blood on paper, and a bench-top spectrophotometer validated the approach before the development of a low-cost reader. We optimized impregnating paper with chemicals to lyse red blood cells, paper type, drying time, wavelengths measured, and sensitivity to variations in volume of blood, and we validated our approach using patient samples. Lysing the blood cells with sodium deoxycholate dried in Whatman Chr4 chromatography paper gave repeatable results, and the absorbance difference between 528 nm and 656 nm was stable over time in measurements taken up to 10 min. after sample preparation. The method was insensitive to the amount of blood spotted on the paper over the range of 5 μL to 25 μL. We created a low-cost, handheld reader to measure the transmission of paper cuvettes at these optimal wavelengths. Training and validating our method with patient samples on both the spectrometer and the handheld reader showed that both devices are accurate to within 2 g/dL of the HemoCue device for 98% and 95% of samples, respectively.

4.2. Introduction

Anemia affects a quarter of the world’s population (1.62 billion people) and can be caused by iron deficiency, malnutrition, blood loss, and infectious diseases (e.g. malaria, hookworm, tuberculosis, HIV), among others. Anemia can cause delayed mental and physical development, fatigue, decreased work productivity, and increased risk of mortality, especially during childbirth. The condition is diagnosed by measuring the concentration of
hemoglobin in the blood. The threshold hemoglobin concentration below which a patient is considered anemic varies by lab and patient condition, but is approximately 11 g/dL for young children and pregnant women, 12 g/dL for non-pregnant women, and 13 g/dL for men (WHO hemoglobin thresholds for people living at sea level). Once the condition is diagnosed, the underlying cause can be determined and treated with, for example, iron supplements, anti-malarial drugs, or blood transfusions.

Hemoglobin concentration assessment is the most commonly performed laboratory test worldwide and is necessary for all healthcare systems. Assessment at the point-of-care enables clinicians to make rapid decisions about treatment. The gold standard for measuring hemoglobin concentration converts hemoglobin to cyanmethemoglobin, a stable variant, and uses a spectrometer to measure its absorbance at 540 nm. This method is not appropriate for use at the point-of-care.

The HemoCue method draws blood into a plastic cuvette and makes spectroscopic measurements to return a hemoglobin concentration. A comprehensive assessment of six hemoglobin concentration tests in Malawi, taking into account accuracy and clinical usefulness, user friendliness, learning and usage time, and economic costs, revealed that HemoCue was the most appropriate method and the recommended standard-of-care in all Malawian district hospitals and urban health centers. The HemoCue method has two associated costs: a fixed cost for the device ($470 from MM African Technologies Ltd in Malawi or $800 from HemoCue, Inc. in the U.S.) and a recurring cost for each cuvette ($0.99 from MM African Technologies Ltd. in Malawi or $1.43 from HemoCue, Inc. in the U.S.). Though the fixed cost is significant, it is primarily the high recurring cost of consumables that prevents widespread utilization of this accurate and easy-to-use hemoglobin
concentration test in low-resource areas where anemia prevalence is highest. The cost of just one cuvette (excluding treatment for anemia, if diagnosed) represents 3% of Malawi’s per capita total expenditure on health per year ($26 in 2010 at an average exchange rate, WHO Global Health Observatory Data Repository). Thus, there is a need for a low-cost alternative that can reduce the consumable component of the per-test cost of assessing hemoglobin concentration at the point-of-care.

4.2.1. Chromatography Paper Alternative

Recently, filter or chromatography paper has been proposed as a low-cost, rugged, and self-contained medium for microfluidic assays in point-of-care systems suitable for developing countries. The ubiquitous and low-cost nature of chromatography paper represents an attractive alternative to expensive plastic cuvettes used in systems such as HemoCue. The goal of this study is to investigate if chromatography paper can serve as a low-cost medium for accurate spectrophotometric detection of blood hemoglobin concentration. We further demonstrate a prototype low-cost device to measure transmission through this chromatography paper “cuvette” and display the hemoglobin concentration at the point-of-care.

4.3. Materials and Methods

In order to validate a paper-based spectrophotometric hemoglobin assessment, we performed experiments to optimize or evaluate sensitivity to five parameters: impregnating paper with chemicals to lyse red blood cells, paper type, drying time, wavelengths measured, and volume of blood. Blood and plasma samples were obtained via venous draw from healthy donors who gave informed consent; plasma was also purchased from the Gulf Coast
Regional Blood Bank (Houston, TX). Protocols were reviewed and approved by the Institutional Review Board at Rice University. For these experiments, whole blood was diluted with plasma to simulate anemia. Micropipettes were used to spot blood on paper to simulate touching a patient’s fingertip to the paper after a fingerprick. A benchtop spectrophotometer (Cary 5000 UV-VIS) recorded the spectra of the samples via an on-axis collimated transmission measurement. The samples were masked with a 3 mm x 3 mm aperture. We used the HemoCue 201+ as a reference standard to develop and evaluate the paper-based approach. The HemoCue 201+ is CLIA (Clinical Laboratory Improvement Amendments) waived and approved for use in the United States by the FDA. HemoCue, Inc. reports an accuracy of ± 1.5% for the HemoCue 201+ when compared to the International Council for Hematology (ICSH) method. Gehring et al. have reported the HemoCue 201+ to be accurate to within approximately 0.5 g/dL of the reference method in a laboratory setting. Paddle et al. have reported 95% limits of agreement of –1.16 g/dL to 0.16 g/dL in a laboratory setting, and Patel et al. have reported a mean difference of 0.110 ± 0.524 g/dL from the reference standard in a hospital setting. Our own measurements provide evidence of the precision of the HemoCue. Values from the spectrum of blood spotted on paper were correlated with the hemoglobin concentration of the sample obtained with a HemoCue. Finally, using conclusions from experiments performed on the spectrometer, we developed a portable, low-cost reader to replace the spectrometer in the field.

4.3.1. Red Blood Cell Lysis

We examined unlysed blood samples as well as samples where red blood cells were mechanically lysed or chemically lysed with sodium deoxycholate. To achieve mechanical
lysis, unlysed blood was taken through multiple (≥3) freeze-thaw cycles (-20°C to 20°C). The blood was considered lysed if high-speed centrifugation did not separate the blood into plasma and red blood cell layers and microscopy showed no intact cells.

To achieve chemical lysis of blood spotted on Whatman Chr4 paper, we used the detergent sodium deoxycholate. To evaluate the amount of sodium deoxycholate needed to achieve lysis on paper, paper strips were treated with various volumes (10, 20, 30, 40, and 50 μL) of 2% or 4% (w/v) sodium deoxycholate in PBS. After the sodium deoxycholate dried, 10 μL of unlysed blood was applied to the treated paper. Unlysed blood and mechanically lysed blood applied to untreated paper served as controls. The blood dried for 2 min and was then eluted from the paper in 1 mL PBS for 10 min. The paper was removed and the spectrum of the remaining solution was measured on a Cary 5000 UV/VIS spectrophotometer from 450 nm to 800 nm. Blood was considered lysed if its transmission spectra did not show evidence of the turbidity associated with intact red blood cells. 10 μL of 4% (w/v) sodium deoxycholate was chosen for all further experiments because (1) it resulted in effective lysis and (2) the blood spread quickly and evenly on the paper without pooling.

To assess the effect of red blood cell lysis on the accuracy of hemoglobin concentration derived from spectral measurements, we measured transmission spectra of samples with varying concentrations of hemoglobin for three conditions: unlysed blood applied to untreated Chr4 paper, mechanically lysed blood applied to untreated Chr4 paper, and unlysed blood applied to sodium deoxycholate-treated Chr4 paper. Blood from 4 donors was diluted with plasma to obtain a range of hemoglobin concentrations spanning the physiologic range. The experiment was repeated on 2 days for each condition; half of the
data were used as a training set, and the remaining data were used as a validation set. The training set for each condition was used to develop an algorithm relating hemoglobin concentration as measured by HemoCue to the absorbance difference at two wavelengths as described in detail in the section “Training and Validation with Patient Samples.” This relationship was used to calculate the hemoglobin concentration of samples in each validation set, and the calculated hemoglobin concentrations were compared to the concentrations obtained from HemoCue. The deviations from HemoCue were binned in 0.1 g/dL increments, and the cumulative percentage of samples with a given deviation was plotted for the three conditions.

4.3.2. Choice of Paper

Seven filter and chromatography papers from Whatman (chromatography papers Chr1, Chr3MM, Chr4, and grades 2, 4, 5, and 6 filter paper) were evaluated for (1) the qualitative appearance of uniform spreading of unlysed blood spotted on sodium deoxycholate-treated paper and (2) the repeatability of spectrophotometric measurements of these blood samples with high and low hemoglobin concentrations. Paper strips were treated with 10 μL 4% (w/v) sodium deoxycholate in PBS to achieve lysis and allowed to dry. 10 μL of unlysed blood was applied and spectra were taken 2 min after spotting. For each type of paper, three measurements were made of blood with a low hemoglobin concentration (7-8 g/dL) and three of blood with a high hemoglobin concentration (15-16 g/dL). Among the papers where blood spread quickly and with little pooling, the measurements of Chr4 had the most repeatable measurements (coefficient of variation of 0.04 for low hemoglobin concentrations and 0.06 for high hemoglobin concentrations) (data not shown). Chr4 was used for all other experiments.
4.3.3. Choice of Wavelength and Time

We determined which spectral measurements could be used to calculate consistent hemoglobin concentrations even if the drying time of the spot varied, an important factor for point-of-care use. To determine the effects of drying time and to select optimal wavelengths for determining hemoglobin concentration, absorption spectra of unlysed blood spotted on sodium deoxycholate-treated Chr4 paper were collected from 450 nm to 800 nm over the course of 30 min.: the time during which a hemoglobin concentration assessment could reasonably be performed in the field. Blood from one donor was diluted with plasma to obtain four hemoglobin concentrations approximating the physiological range. Each concentration was measured three times, and spectra were taken every 2 min.

4.3.4. Effects of Blood Volume

Absorption spectra of various volumes (5, 10, 15, 20, 25 μL) of unlysed blood on sodium deoxycholate-treated Chr4 paper were obtained to determine the effects of blood volume on the test. Three hemoglobin concentrations were examined, and three measurements were made of each concentration at each volume. These transmission spectra were used to calculate hemoglobin concentrations with an algorithm developed using data from a training set of samples described in the section “Training and Validation with Patient Samples;” we examined whether the calculated concentration of hemoglobin depended on sample volume.

4.3.5. Training and Validation with Patient Samples

An algorithm was developed to calculate hemoglobin concentration from on-axis transmission measurement of blood spotted on paper. Patient blood samples were used to
train and validate this algorithm on the laboratory spectrometer. Whole blood samples were obtained from 48 hospitalized patients. Venous blood was collected in heparinized tubes; anonymous specimens were obtained one week after collection. The protocol was reviewed and approved by the Rice University IRB and was found to be exempt from IRB review. 5 patient samples were discarded because the blood showed significant clotting. 2 measurements were made from each patient sample. Data from 21 patients (42 measurements) were used to develop an algorithm relating the hemoglobin concentration as measured by HemoCue to the absorbance difference between two wavelengths using a best-fit power curve:

\[
[Hb] \text{ in g/dL} = A \cdot [\text{Extinction coefficient}(\lambda_1) \ - \ \text{extinction coefficient}(\lambda_2)]^n
\]

where \(A, \lambda_1, \lambda_2\), and \(n\) were varied.

These wavelengths include one wavelength from 450 nm to 600 nm where hemoglobin absorbs and one wavelength from 600 nm to 800 nm where it does not absorb. As described in the results section, the pair of wavelengths that gave the best results and was least sensitive to variations in time between sample preparation and sample measurement was 528 nm and 656 nm. Data from the remaining 22 patients (44 measurements) were used to validate the performance of this algorithm. The hemoglobin concentration for these samples was calculated from the transmission data using the relationship from the training set; results were compared to those measured by the HemoCue. For both training and validation sets, a reading was taken on the spectrometer at 2 min. after spotting.
4.3.6. Development of a Low-Cost Reader

Figure 4-1. Photograph (a) and light path schematic (b) of self-contained low-cost reader.

Following validation of the algorithm to calculate hemoglobin concentration on a spectrometer, we developed a prototype low-cost reader to replace the spectrometer (Figure 4-1). A diagram of the optical components of the low-cost reader is presented in Figure 4-1b. Light emitted from two LED diodes (HyperRed, $\lambda = 656 \pm 25$ nm, 720-LHW5AM1T3T1LZ from Osram via Mouser Electronics and TrueGreen, $\lambda = 528 \pm 33$ nm, 720-LTW5SMJXKX36Z from Osram via Mouser Electronics) is collimated by plastic aspheric lenses (EFL=3.3mm, NA=0.4, CAY033 from ThorLabs) and, after passing through 50/50 beamsplitter (BS007 from ThorLabs), is directed onto the sample. Light transmitted through the sample is detected by a broad-band photodiode (wavelength range: 350-1100 nm).
nm, FDS100 from ThorLabs) placed behind the sample chamber. All electronic components of the system are connected to an Arduino Nano 3.0 microcontroller. A custom-written Arduino program was used to acquire and analyze measurement data. Measurements consist of a 2 sec. baseline reading with both LEDs off to enable subtraction of ambient light, a 2 sec. reading with the 528 nm LED on, and a 2 sec. reading with the 656 nm LED on. Results are displayed on a three digit numeric display (Kingbright BC56-12SRWA), and the prototype is powered by a rechargeable 9V battery. The device measures 6.4 cm x 6.6 cm x 7.8 cm and weighs 281 g.

Samples are inserted between two custom 3D-printed plastic pieces to isolate the reader from biohazards. These pieces may be reused for many samples and are sized such that they may be replaced by glass slides. The chromatography paper is cut such that aligning it with the depression in the plastic holder ensures proper alignment of the blood sample with the optics of the low-cost reader.

Data were collected from the same samples used to train and validate the spectrometer at 90 sec. after spotting. Data from the training set were used to develop an algorithm relating the difference in transmission at 528 nm and 656 nm to the hemoglobin concentration as determined by the HemoCue:

\[
[Hb] \text{ in g/dL} = A \times [\text{Extinction coefficient(} \lambda_1 \text{)} - \text{extinction coefficient(} \lambda_2 \text{)}]^n
\]

where A, \( \lambda_1 \), \( \lambda_2 \), and n were varied.

The algorithm was then used to predict the hemoglobin concentration for the samples in the validation set using the concentration determined with the HemoCue as the
gold standard. The Arduino program was developed to calculate and display the hemoglobin concentration according to this algorithm.

### 4.4. Results and Discussion

#### 4.4.1. Absorbance Spectrum of Blood on Paper

![Absorbance Spectrum of Blood on Paper](image)

Figure 4-2. Extinction coefficient spectrum of unlysed blood on untreated Chr4 paper as calculated from collimated transmission measurement. Hemoglobin concentration as determined by HemoCue was 14.9 ± 0.3 g/dL (solid line) and 2.9 ± 0.0 g/dL (dashed line).

From 450 nm to 600 nm, spectra of blood on paper show characteristic absorbance peaks due to hemoglobin; above 600 nm, spectra show a nearly flat baseline region (Figure 4-2). These spectra of blood on paper (Figure 4-2) compare well with published spectra of hemoglobin in solution\(^7\). We hypothesized that the hemoglobin concentration was related to the difference in optical density between measurements made in the spectral region where hemoglobin absorbs and in the baseline region where it does not absorb.
4.4.2. Red Blood Cell Lysis

A diagnostic tool must give consistent results to be useful. We examined whether hemolysis affected the variability of light transmitted through paper spotted with blood. Figure 4-3a shows the cumulative percentage of samples with a given deviation from HemoCue for unlysed blood, mechanically lysed blood, and chemically lysed blood. Figure 4-3b shows the percent of samples for a given condition that are within ±1 g/dL of the hemoglobin concentration obtained by HemoCue. The highest percentage of samples was within ±1 g/dL when using mechanically lysed blood. On average, accuracy was slightly

![Figure 4-3](image-url)
reduced when using chemically lysed blood. Accuracy was lowest when using unlysed blood. Though chemical lysis with sodium deoxycholate does not give the level of accuracy achieved with mechanically lysis, it represents an improvement over unlysed blood, and chemical lysis with pre-treated paper can be performed easily at the point of care.

We hypothesize that the free hemoglobin molecules in lysed blood spread more evenly throughout the paper than intact red blood cells do. This uniformity in spreading helps ensure that light transmission through the square of paper measured by the spectrometer can be used to accurately determine the hemoglobin concentration for each blood sample.

4.4.3. Wavelength and Drying Time Optimization

To be useful in a busy point-of-care setting, the test should not be overly dependent on the time between sample preparation and sample measurement.

To determine the appropriate wavelengths, absorption spectra from 450 to 800 nm of unlysed blood spotted on sodium deoxycholate-treated paper were measured versus time over the course of 30 min. for samples at four hemoglobin concentrations. Then, the difference in optical density between various pairs of wavelengths was plotted versus time, and the algorithm developed using the training set described in the section “Training and Validation with Patient Samples” was used to calculate the hemoglobin concentration. The algorithm was derived from training set spectra taken 2 min. after spotting; this algorithm was used to calculate hemoglobin concentration at each time point.
As shown in Figure 4-4, the absorbance of hemoglobin on paper was found to vary more with time at some wavelengths than at others. Some pairs of wavelengths give results that are stable over time, such as the difference between 528 nm and 656 nm (an average increase of 1.9 g/dL over 10 min., Figure 4-4a). Other pairs, such as 590 nm and 656 nm, result in increasing hemoglobin concentrations over time (an average increase of 4.9 g/dL over 10 min., Figure 4-4b). The choice of wavelength in the region where hemoglobin does not absorb had little effect on the results (data not shown). Other choices of wavelengths also give consistent results over time (such as 540 nm and 680 nm, with an average increase of 0.9 g/dL over 10 minutes), but 528 nm and 656 nm were chosen to match the availability of...
LEDs for our low-cost reader. The bandwidths of the LEDs (33 nm for the 528 nm LED and 25 nm for the 656 nm LED) include these other stable choices of wavelength.

Using 528 nm and 656 nm for calculation of hemoglobin concentration allows a healthcare worker to most accurately obtain a hemoglobin concentration without precisely monitoring the time from sample preparation to measurement, as long as the sample is measured within 10 min. For the remainder of the experiments, spectra were taken at 2 min. after spotting, and the absorbance difference between 528 nm and 656 nm was used to calculate hemoglobin concentration and compare to results determined by HemoCue.

4.4.4. Volume Effects

Because the volume of blood in a finger-prick can vary, we examined how sensitive the method is to changes in blood spot volume. The absorbance difference between 528 nm and 656 nm was measured for various volumes of blood from three blood samples of different hemoglobin concentrations (Figure 4-5).
The calculated hemoglobin concentrations for all volumes tested (5 μL to 25 μL) are not statistically different (p = 0.82 for the high hemoglobin concentration, p = 0.37 for the middle hemoglobin concentration, and p = 0.15 for the low hemoglobin concentration), so this range of volumes is considered appropriate for measurements. Volumes of 2.5 μL were also evaluated, but this volume of blood was not large enough to fill the 3 mm x 3 mm aperture used in the spectrometer and thus gave highly variable results.

To help health workers gauge that a sufficiently large volume has been spotted on the paper, eight small dots were laser printed onto the paper in the shape of a circle with diameter ~7.7 mm. This circle holds approximately 10 μL of blood. This circle also denotes where the sodium deoxycholate lysing agent has been dried onto the paper.

Figure 4-5. Calculated hemoglobin concentration from three samples with different volumes of blood spotted on sodium deoxycholate-treated paper. All samples of a given hemoglobin concentration were from one blood donor. Hemoglobin concentration was calculated using the training set shown in Figure 4-6. Error bars represent one standard deviation for the average of 3 samples. HemoCue determined the concentration of the samples to be 15.0 ± 0.1 g/dL, 9.8 ± 0.1 g/dL, and 5.3 ± 0.1 g/dL.
Figure 4-6. Training (solid circles) and validation (open diamonds) sets for the spectrometer (a) and the low-cost reader (b) using patient blood samples. Training, \( n = 44 \), validation, \( n = 42 \). Fit line and equation shown are for the training set. Hemoglobin concentration was determined by the average of four measurements on the HemoCue; vertical error bars represent one standard deviation of these measurements. Horizontal error bars represent one standard deviation of two replicate measurements on the spectrometer (a) and low cost reader (b). Bland-Altman plots comparing the HemoCue to the hemoglobin values calculated for the validation set by the spectrometer (c) and the low-cost reader (d).
Results from the training and validation sets for both the spectrometer and the low-cost reader are shown in Figure 4-6. The Bland-Altman plots (Figure 4-6 c,d) show that the approach is accurate to within ±2 g/dL of the HemoCue device for 98% (spectrometer) and 95% (low-cost reader) of samples. Our 95% limits of agreement are -1.91 g/dL to +2.10 g/dL for the spectrometer and -2.07 g/dL to +1.86 g/dL for the low-cost reader. It should be noted that none of the samples from hospitalized patients had a hemoglobin concentration higher than the normal range of hemoglobin concentrations for adult men (an upper bound of approximately 18.5 g/dL).

Gomez-Simon et al. tested the HemoCue against a laboratory hematology analyzer in a mobile blood donation setting and found 95% limits of agreement of -0.68 g/dL to +2.25 g/dL. This point-of-care setting may be comparable to our targeted setting.

The WHO hemoglobin color scale also uses blood spotted on paper to determine hemoglobin concentration. With this method, the color of a blood spot is visually compared to reference standards ranging from 4 g/dL to 14 g/dL in gradations of 2 g/dL. This method is prone to inaccuracies: van den Broek et al. reported that the color scale was within 2 g/dL of a laboratory hemoglobin value in only 67% of cases. Paddle, using one observer in ideal conditions, found the 95% limits of agreement for the color scale to be -3.50 g/dL to +3.11 g/dL. The color scale strips are available in Malawi for ~$0.02/test. We have summarized the reported accuracy and cost of these methods in Table 4-1. Our spectrophotometric method has the potential to provide increased accuracy over the current WHO color scale-based method with comparable or reduced cost. The accuracy of our method does not meet the ±7% agreement limits set by CLIA.
While these results are encouraging, our study has a number of limitations. Our method does not give perfectly repeatable measurements for a given sample of blood. We hypothesize that these inconsistencies arise due to a combination of variations in paper thickness and fiber arrangement or slight variations in how the blood was applied to the paper. We may be able to increase the accuracy in further studies by applying reagents to the paper to convert oxy- and deoxyhemoglobin to more chemically stable forms, such as the conversion to azide methemoglobin that is performed inside the HemoCue cuvettes. However, at present, the approach provides a more accurate, lower-cost method than is currently available with the WHO hemoglobin color scale and approaches the accuracy of the HemoCue in a mobile blood donation setting. In this study, blood was applied with a

Table 4-1. Accuracy and Cost of Three Methods for Determining Hemoglobin Concentration

<table>
<thead>
<tr>
<th>Technique</th>
<th>Setting</th>
<th>Reported Accuracy (95% agreement limits unless otherwise stated)</th>
<th>Per-Test Cost</th>
<th>Reader Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>HemoCue</td>
<td>Laboratory</td>
<td>- 0.5 to + 0.5 g/dL&lt;sup&gt;4&lt;/sup&gt;</td>
<td>$1.43 (US)</td>
<td>$800 (US)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$0.99 (Malawi)</td>
<td>$470 (Malawi)</td>
</tr>
<tr>
<td>HemoCue</td>
<td>Field</td>
<td>- 0.68 to + 2.25 g/dL&lt;sup&gt;8&lt;/sup&gt;</td>
<td>$1.43 (US)</td>
<td>$800 (US)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$0.99 (Malawi)</td>
<td>$470 (Malawi)</td>
</tr>
<tr>
<td>WHO Color Scale</td>
<td>Laboratory</td>
<td>- 3.50 to + 3.11 g/dL&lt;sup&gt;5&lt;/sup&gt;</td>
<td>$0.02 (Malawi)</td>
<td>$8 (Malawi)</td>
</tr>
<tr>
<td>WHO Color Scale</td>
<td>Field</td>
<td>67% of measurements within 2 g/dL of reference</td>
<td>$0.02 (Malawi)</td>
<td>$8 (Malawi)</td>
</tr>
<tr>
<td>Spectrophotometric</td>
<td>Laboratory</td>
<td>- 2.07 g/dL to +1.86 g/dL</td>
<td>$0.006</td>
<td>$400</td>
</tr>
<tr>
<td>measurement of blood spotted on</td>
<td></td>
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</tbody>
</table>
micropipette and volume was controlled. A field test must be conducted where blood samples are collected directly from a fingerprick to evaluate the effects of a different method of application and of varying volumes of blood. In addition, the method has been validated with a relatively small sample size, which may not include patients with other hemoglobinopathies or blood disorders that could affect accuracy. Finally, while the HemoCue 201+ is approved for clinical use, it is not as accurate as laboratory reference methods based on spectrophotometry. The use of HemoCue as a reference standard may have biased estimates of the accuracy of the paper-based method described here. Further work is necessary to determine the impact of the potential sources of errors in the reference standard.

4.4.6. Cost Analysis and Biohazard Risk Reduction

Whatman Chr4 chromatography paper, cut to 2.5 cm x 2.5 cm and combined with the necessary sodium deoxycholate, was calculated to have an approximately 99% decrease in cost when compared to HemoCue cuvettes. Pricing for HemoCue cuvettes is $1.43/cuvette (as of January 2013) when purchased directly from HemoCue, Inc. in the United States and $0.99/cuvette (as of January 2013), when purchased from MM African Technologies Ltd. in Malawi. In comparison, each 2.5 cm x 2.5 cm strip of Whatman Chr4 costs $0.006 when bought from Sigma-Aldrich. 10 μL of 4% (w/v) sodium deoxycholate contains 0.4 mg of sodium deoxycholate, giving a per-test cost for the reagent of $0.0003 when purchased from Sigma-Aldrich. Thus, the total material cost for the hemoglobin strip is $0.0063, which is a 99.6% reduction in price from HemoCue cuvettes purchased in the United States and a 99.4% reduction from cuvettes purchased in Malawi. It should be noted
these calculations represent only the material cost and do not include costs to manufacture or package the strips.

The plastic HemoCue cuvettes filled with blood necessitate considerations in minimizing biohazard risk. Our paper platform does not require sharps disposal, and it can be easily incinerated on-site to dispose of any potential biohazards.

The cost to build one prototype reader was approximately $400 (see Table 4-2). The reusable plastic holder made for the low-cost device costs approximately $4.00 to prototype on an in-house 3D printer. These calculations again only include the material cost for our device. There is significant potential to reduce this cost when the prototype is produced at higher volumes, particularly the cost of the fixture and the beamsplitter. We estimate that the materials cost of the device can be reduced by approximately one order of magnitude when produced at volumes of 10,000.
4.5. Conclusions

This study demonstrates that chromatography paper is a low-cost medium that can be used for spectrophotometric detection of blood hemoglobin concentration with 98% (spectrometer) and 95% (low-cost reader) of samples within 2 g/dL of the reference. We developed a low-cost reader for the chromatography paper method for use in the field and
validated the spectrometer and low-cost reader with clinical samples. The ability to reduce the per-test cost by ~99% and biohazard risk represents an important development in increasing the availability of a hemoglobin concentration assay in developing countries where anemia is prevalent. While performance must be improved to meet CLIA guidelines, current performance in the laboratory exceeds that reported for the WHO Hemoglobin Color Scale method in similar conditions. Moreover, the approach has the potential to significantly reduce the per-test cost of approaches that rely on more expensive plastic cuvettes. Field studies will be necessary to evaluate accuracy in relevant clinical settings and to establish whether the approach is cost-effective.
Chapter 5

Design and Performance of a Low Cost, Handheld Reader for Diagnosing Anemia in Blantyre, Malawi\textsuperscript{3}

5.1. Abstract

Anemia, a condition characterized by insufficient hemoglobin, affects 56.2\% of pregnant women and 66.1\% of children under five in low-resource countries. Though hemoglobin concentration measurement is the most common laboratory test in the world, the high cost of disposables (>\$1.00 per test in Malawi) limits its availability in these settings. We have

\textsuperscript{3} The contents of this chapter have been published in the following conference proceedings: Bond M, Mvula J, Molyneux E, and Richards-Kortum R. Design and Performance of a Low-Cost, Handheld Reader for Diagnosing Anemia in Blantyre, Malawi. Health Innov Point Care Conf. 2014; 267-270. doi: 10.1109/HIC.2014.7038926. © 2014 IEEE. Reprinted with permission.
demonstrated a spectrophotometric method that reduces the per-test cost of anemia diagnosis to under $0.01 by using chromatography paper as the only disposable.

Improvements in the hand-held reader, including using laser modules and a reference photodiode, enabled repeatable results within and across devices. We evaluated this method by analyzing capillary blood samples from 70 patients in the pediatric ward of Queen Elizabeth Central Hospital, Blantyre, Malawi. ~90% of these samples were within 2 g/dL of the standard value, with higher accuracy on more anemic samples. Current work aims to improve this accuracy by converting the hemoglobin in the sample to the more stable form methemoglobin.

5.2. Introduction

Anemia, an insufficient concentration of hemoglobin, can lead to decreased productivity, fatigue, delayed development, and an increased mortality risk. It is diagnosed by measuring the concentration of hemoglobin in the blood and can be treated with iron supplementation, blood transfusions, or treating an underlying infection such as malaria. In countries classified as least developed by the UN Human Development Index, anemia affects 66.1% of preschool-aged children and 56.2% of pregnant women. In Malawi, it is estimated that 73.2% of the population has hemoglobin levels below 11 g/dL. The cutoff hemoglobin concentration for defining anemia depends on the patient’s age, sex, and location, but is generally between 11 and 13 g/dL for people living at sea level. Despite the great need for accurate anemia diagnostics, the high cost of disposables prevents widespread use of methods like the HemoCue 201+ (approximately $1.00/cuvette when purchased in Malawi).
Previously, we have demonstrated a low-cost method for hemoglobin concentration assessment using treated chromatography paper as the only disposable (<$0.01/test)\(^2\). A low-cost, handheld reader measures the paper cuvettes. Briefly, blood is applied to the chromatography paper where the cells are lysed with dried sodium deoxycholate. The paper is placed inside a reusable plastic holder and inserted into the reader. The handheld, battery-powered reader ("HemoSpec") measures the transmission of the blood at a green wavelength (where hemoglobin absorbs strongly) and a red wavelength (where hemoglobin does not absorb strongly). The difference in absorbance of the blood between the green and red wavelengths correlates with the hemoglobin concentration as determined by a reference standard.

The handheld reader has been modified to give more reliable results and a field trial has been conducted in Blantyre, Malawi to assess the accuracy of the method.

### 5.3. Methods

#### 5.3.1. Device

In preparation for field trials of the method, we modified the device to give more reliable device-to-device readings, to be easy for users to calibrate, and to measure more accurately the optical density of the sample.

In order to improve device-to-device reliability, we used laser modules instead of LEDs and improved optical component mounts so that devices were less sensitive to misalignment during transport. Laser modules (532 nm DPSS-B from Z-Bolt, 650 nm 381003-ND from DigiKey) have a low divergence angle (1.2mrad and 1.6mrad, respectively),
and thus the beams do not interact with the walls of the optical holder like the beams of wide-angle LEDs do. The optical components (laser modules, beam splitter BS010 from ThorLabs, photodetectors FDS100 from ThorLabs, and custom-made aluminum apertures) were held securely in two halves of a 3D printed holder (Figure 5-1). The two halves of the holder were bolted together to minimize movement of the parts between measurements. A second, reference detector was added to correct for any temporal variation in the laser output.

![Image](image1.png)

Figure 5-1. (a) Photograph of assembled HemoSpec. (b) Schematic of optical holder. Light comes from green and red laser modules, through a 50/50 beamsplitter, and either into a reference photodetector (R) or through the sample and into the sample photodetector (S). The reference detector is protected from intense green light with red cellophane. Slots around sample indicate space for aluminum apertures. Four holes allow the two halves of the optical holder to be bolted together securely.

The optical density of the sample is calculated as

$$OD = -\log_{10}\frac{\text{transmitted}}{\text{incident}},$$

(1)

where \(\text{transmitted}\) is the voltage measured by the sample detector and \(\text{incident}\) is the adjusted voltage measured by the reference detector. The detectors have different levels of gain due
to the reduced amount of light reaching the sample detector, so the incident signal must be corrected to be comparable to the signal received by the sample detector.

Correction factors are calculated during calibration, in which the user inserts a series of three neutral density filters of known optical density following prompts on the device screen. Calibration takes less than 1 minute and should be performed at the start of each day’s measurements or when the device is moved to a new location.

Additionally, apertures were added to the optical train to enable more accurate calculation of optical density. Because paper is highly scattering, without apertures, a detector placed close to the sample receives more light (transmitted plus scattered) than a detector placed far away from the sample. We added two 3 mm diameter apertures placed 1 mm from the sample and 6 mm from each other to reduce the amount of scattered light that reaches the sample detector. A 5 mm diameter aperture was placed 1 mm before the sample to ensure that all light passes through the region of the paper where blood has been applied.

The assembled unit is controlled with a custom-programmed Arduino Nano microcontroller (Gravitech, Minden, NV, USA), is powered with a rechargeable 7.4V lithium-poly battery, and displays results on a 16 character LCD screen.

5.3.2. Malawi

To test this method and device in the field, we recruited 70 patients from the pediatric ward of the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi. The protocol was approved by the Rice IRB and Malawi COMREC. Patients aged 0-15 years were eligible for the study if the treating physician requested a hemoglobin concentration measurement and the parents provided informed consent.
Previous research has shown significant variability in the hemoglobin concentration of capillary blood drawn simultaneously from the right and left hands \(^{26}\) and as compared to venous blood\(^{21}\). Our own experiments (data not shown) have suggested that there is also significant variation in the hemoglobin concentration of successive drops of blood collected from a fingerprick. To remove these effects from our assessment of the accuracy of the device, we collected approximately 20 \(\mu\)L of blood from a fingerstick into an EDTA-coated collection tube. This blood was thoroughly mixed with a pipette, and then 10 \(\mu\)L was measured using the HemoSpec and 10 \(\mu\)L using the HemoCue 201+ (HemoCue AB, Ängelholm, Sweden), our gold standard. Both devices thus received blood with the same hemoglobin concentration.

The HemoCue 201+ is an appropriate gold standard for this study in a low-resource setting because it was portable, battery-powered, and required only cuvettes to operate. Numerous studies have demonstrated the accuracy of the HemoCue in the lab\(^{33,36,37}\), and our own measurements (data not shown) demonstrate that the HemoCue compares well to an Act-Diff 2 hematology analyzer (Beckman Coulter, Irving, Tx, USA) using well-mixed venous blood in the lab.

For analysis on the HemoSpec, 10 \(\mu\)L of whole blood were pipetted directly onto a square of chemically treated chromatography paper contained in a reusable plastic holder. The holder and blood-spotted paper were then placed into the HemoSpec, which returns a hemoglobin concentration in approximately 20 seconds.

70 samples were collected in Malawi. 19 samples were removed from analysis due to clotting, insufficient volume, or sitting for more than approximately 30 minutes before measurement, leaving 51 samples for analysis.
5.4. Results/Discussion

5.4.1. Device

Table 5-1. Repeatability Across Devices. Data represent average ± standard deviation for 5 measurements.

<table>
<thead>
<tr>
<th>Neutral Density Filter</th>
<th>OD at 532 nm – OD at 656 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Device 1</td>
</tr>
<tr>
<td>2.5 OD</td>
<td>0.18 ± 0.00</td>
</tr>
<tr>
<td>3.0 OD</td>
<td>0.14 ± 0.00</td>
</tr>
</tbody>
</table>

To assess the consistency of results across multiple devices, neutral density filters were measured on all devices. Table 5-1 shows the mean and standard deviation of five measurements of the difference between the optical density evaluated at 532 nm and 656 nm made by three different devices for a 2.5 and 3.0 OD neutral density filter. (Neutral density filters do not have a perfectly flat spectrum, so there is a measurable difference in optical density between 532 nm and 656 nm.) A change of 0.02 in difference of optical density (for example, the difference between devices 1 and 2 measuring the 2.5 OD filter) corresponds to a change in reported hemoglobin concentration of 0.09 g/dL, a clinically insignificant change.
Figure 5.2 compares the difference in optical density (OD at 532 nm – OD at 650 nm) as determined by the HemoSpec to that determined by a Cary 5000 UV-VIS spectrophotometer for 34 blood samples of varying hemoglobin concentration spotted onto chromatography paper. The two apertures after the sample in the HemoSpec reduce the amount of scattered light reaching the sample detector, and thus give an optical density comparable to that measured by the spectrophotometer, which measures almost exclusively transmitted light. A good concordance between OD measurements on the two devices allows for easy verification of the HemoSpec’s performance without concern for the effect of the device performing the measurement.

The materials cost for one HemoSpec is approximately $450, including materials for 3D-printing the plastic holder. This cost can decrease considerably with bulk production.
5.4.2. Malawi

Results from the study in Malawi are shown in Figure 5-3. Figure 5-3a compares the hemoglobin concentration determined by the HemoSpec to that determined by the HemoCue (standard). Figure 5-3b shows these results on a Bland-Altman plot. As seen in Fig. 3c, 86% of samples are within 2 g/dL of the reference standard. This value correlates well with the 95% of samples within 2 g/dL in an assessment of the method performed in laboratory conditions².
Figure 5-3. (a) Hemoglobin concentration determined by the HemoSpec and the HemoCue (standard). Solid line represents equivalence; dotted lines represent ± 2 g/dL deviation from equivalence. (b) Bland-Altman plot showing the average of hemoglobin concentration determined by each device against the difference in hemoglobin concentration between the two devices. (c) Histogram showing the number of samples (left axis) and the cumulative percent of samples (right axis) achieving the given deviation from the standard. (d) Breakdown of the cumulative percent of (c) according to hemoglobin concentration as determined by the standard. n = 5 for 4–8 g/dL (open diamonds), n = 30 for 8–12 g/dL (solid squares), and n = 16 for > 12 g/dL (shaded).

Figure 5-3d shows the cumulative percent of samples reaching a given deviation from the standard, broken down by hemoglobin value as determined by the standard. These results suggest that the method is more accurate for more anemic samples, though analysis with a greater number of samples is necessary (n = 5 for 4–8 g/dL, n = 30 for 8–12 g/dL, and n = 16 for > 12 g/dL). Clinicians at QECH use a cutoff of 5 g/dL when deciding to transfuse.
a patient, so higher accuracy around this value is desirable. We believe this higher accuracy at lower concentrations is due to the more even spreading of anemic blood on paper as compared to normal blood and the lower optical density of anemic blood at 532 nm.

5.5. Conclusion

We have demonstrated a reliable, low-cost handheld reader for anemia diagnosis. The HemoSpec readers give consistent results on different devices for standard samples. The difference in optical density between 532 nm and 656 nm determined by the HemoSpec for samples of blood on paper corresponds well to the difference in optical density determined by a lab spectrophotometer.

The HemoSpec method was tested with 70 fingerstick blood samples in the Queen Elizabeth Central Hospital of Blantyre, Malawi, and achieved 86% of samples within 2 g/dL of the standard method. This accuracy was higher for more anemic samples.

While 2 g/dL accuracy in the field represents better accuracy than other low-cost methods of hemoglobin determination such as the WHO color scale, it is not sufficient for clinical use. Clinicians in Malawi request accuracy of within 1 g/dL of a standard, and in the United States, CLIA (Clinical Laboratory Improvement Amendments) requires ±7% accuracy. We are working to improve the accuracy by incorporating chemistry into the disposable to convert the oxy- and deoxyhemoglobin into more stable methemoglobin. Preliminary results suggest that achieving these improvements in a single paper cuvette will improve our accuracy to within 1 g/dL of the standard value. Further field studies will assess the accuracy of this improved method.
Chapter 6

Nitrocellulose Paper Accurately Measures Hemoglobin Concentration

6.1. Abstract

Anemia is most common in the regions of the world least able to afford effective diagnostics, affecting more than 60% of children under five years old in much of sub-Saharan Africa. Clinicians need a hemoglobin measurement system that can be used at the point of care, is accurate, easy to use, and affordable. Current methods to measure hemoglobin suffer from a high per-test cost or low accuracy. Previously, we demonstrated a low-cost, point-of-care method to diagnose anemia by spotting a drop of fingerprick blood on chromatography paper and using a portable reader to measure optical transmission at two wavelengths. Here, we demonstrate improved precision by using nitrocellulose paper (3.8-

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4 This chapter was prepared with the assistance of Maryam Nemati, Kathleen Schmeler, Alyssa Rieber, and Rebecca Richards-Kortum and will be submitted for publication.
fold reduction in %CV) and prolonged window of time for accurate measurement from sample collection (from 2 to 22 min), while maintaining a low per-test cost (approximately $0.016 per test). We evaluated the accuracy of the method using diluted donor blood in a laboratory setting and found that 98% of samples were within ±1 g/dL of the reference method. We evaluated the accuracy of the method using fingerprick blood from 49 oncology patients at Lyndon B. Johnson Hospital in Houston, TX, achieving 89% of samples within ±1 g/dL of the reference method.

6.2. Introduction

Clinicians in low-resource settings need a low-cost, accurate method to diagnose anemia at the point of care. Anemia (a low blood hemoglobin concentration) can be caused by iron deficiency, blood loss, inherited disorders like sickle cell disease, and infectious diseases like malaria and HIV$. Left untreated, it can result in delayed motor and cognitive development in children, fatigue, low productivity, and increased risk of maternal and fetal death during pregnancy and childbirth$. Once the condition is diagnosed, it can be treated by addressing the underlying cause with, for example, iron supplementation, blood transfusion, or anti-malarials.

Anemia is most common in the regions of the world least able to afford effective diagnostics, affecting more than 60% of children under five years old and 45% of pregnant women in much of sub-Saharan Africa$^{39}$. To diagnose and treat anemia, clinicians need a hemoglobin measurement system that can be used at the point of care, is accurate, easy to use, and low cost. While the capital or reader cost should be low, it is often the per-test cost that determines the long-term usefulness of a test in low-resource settings$^8$. 
Many commercial methods exist to determine hemoglobin concentration at the point of care. A review of six methods in Malawi identified the HemoCue as the most appropriate device for this setting because of its high accuracy (accurate to within approximately 0.5 g/dL of a reference standard\textsuperscript{33,37}) and user friendliness, though the cost (US$0.75/test in 2005) was too high for wide-spread use in low-resource settings\textsuperscript{8}. The cost of each single-use HemoCue cuvette as of 2015 is US$1.53 in the United States and approximately US$1.00 in Malawi, where the yearly per-capita health expenditure was only US$26 in 2013, according to the World Bank. Low-cost methods like the WHO Haemoglobin Colour Scale (HCS, US$0.03/test) exist, but suffer from low accuracy. In a systematic review, Critchley and Bates found the HCS had 75-97% sensitivity and 41-98% specificity for detecting anemia\textsuperscript{40}. Paddle found that only 46% of HCS measurements were within 1.0 g/dL of the reference method\textsuperscript{36}. Thus, there exists a need for a test that has both a low per-test cost and high accuracy.

To meet this need, a number of point-of-care hemoglobin tests are in development. Yang et al. reported a method where 20 μL of blood are mixed with Drabkin reagent, spotted onto chromatography paper, allowed to dry, and measured on a flat-bed scanner.\textsuperscript{41} They report 90.7% of samples within 1 g/dL of the reference, but the sequence takes 35 minutes to report an answer and requires a large number of user steps. Tyburski et al. have developed a plastic device to collect blood and mix it with a reagent solution that gives a colorimetric readout (blue to red) correlating with the hemoglobin concentration of the sample\textsuperscript{42}. The device appears appropriate for qualitatively assessing anemia status at the point of care, but the low quantitative accuracy (95% limits of approximately ±3 g/dL) and the high per-test cost of $0.50 limit its use for clinical settings in low-resource areas.
Previously, we demonstrated a low-cost method to diagnose anemia at the point of care\textsuperscript{2,3}. A drop of blood is spotted on detergent-treated chromatography paper, and a small reader measures the optical density of the paper at a green wavelength (where hemoglobin absorbs strongly) and a red wavelength (where hemoglobin absorbs minimally). The difference in absorbance at these two wavelengths is used to calculate the hemoglobin concentration of the blood. Pilot clinical studies to assess accuracy of the system showed that 95\% of samples were within 2 g/dL of a reference standard in a laboratory setting\textsuperscript{2} and 86\% of samples were within 2 g/dL in a hospital in Blantyre, Malawi\textsuperscript{3}. While the method was easy to use and low cost (<$0.01 per test and approximately $450 for a prototype reader), improved accuracy (deviation of \(\leq 1\) g/dL from a reference standard) is required for use in a clinical setting. In this paper, we describe modifications to the disposable and the reader to increase accuracy and then evaluate the performance of the improved method in the lab and in the field.
6.3. Methods

![Image of HemoSpec reader and disposable cartridges](image)

Figure 6-1. Reader and disposable cartridges. (a) HemoSpec reader. (b) Nitrocellulose cartridge. (c) To apply the sample, the user removes protective film from clear tape. (d) Approximately 10 μL of blood is applied to the nitrocellulose in the position indicated by the cutouts, and the tape is folded over. (e) The disposable cartridge slides into the reusable sample holder, which is then placed into the reader. Scale bars in (b-e) are 10 mm.

6.3.1. Design of reader

The portable reader ("HemoSpec") has been described previously\(^3\). Briefly, 532 nm and 650 nm laser modules illuminate a 50/50 beamsplitter; light from the beamsplitter is directed to a reference photodiode or through the disposable cartridge to a sample photodiode. Measurements are controlled and interpreted with a custom-programmed
Arduino Nano microcontroller (Gravitech, Minden, NV, USA). A photograph of the device is shown in Figure 6-1a.

To take a measurement, the user peels off the protective backing from the disposable cartridge (Figure 6-1c), applies a drop of blood to the location indicated by the triangular cutouts, allows the blood to absorb (~15 seconds), folds over the tape (Figure 6-1d), slides the cuvette into the reusable sample holder (Figure 6-1e), and places the assembly into the reader. The reusable plastic sample holder is placed inside an L-shaped cutout on the HemoSpec reader (Figure 6-1a). This ensures that the spotted side of the nitrocellulose always faces the sample detector.

To calibrate the device, the user measures a series of three neutral density filters (of OD 2.0, 2.5, and 3.0) and a piece of blank nitrocellulose paper. The device automatically uses data from these standards to calculate correction factors to account for variations in light intensity, detector sensitivity, and changes in optical throughput. The process takes less than two minutes and should be performed at the beginning of each day of use.

6.3.2. Nitrocellulose is more precise than chromatography paper

We compared the precision of light transmission measured when blood was spotted on nitrocellulose and chromatography papers. The same sample of blood was spotted on both types of papers and transmission spectra were measured on a Cary UV-VIS spectrometer. Separate samples were also measured on a HemoSpec reader. EDTA venous blood was diluted with plasma purchased from the Gulf Coast Regional Blood Bank (Houston, TX) to create 2 mL samples with varying hemoglobin concentrations. HF090 nitrocellulose (EMD Millipore, Darmstadt, Germany) was prepared by cutting with a laser
cutter, dipping into 0.04 g/mL sodium deoxycholate in 1x PBS, and allowing to dry. This nitrocellulose has a clear 2 mil plastic backing that allows for easy handling. Whatman Chr4 (GE, Schenectady, New York) chromatography paper was prepared by pipetting 10 μL of 0.04 g/mL sodium deoxycholate in 1x PBS and allowing to dry. 10 μL of blood was pipetted on to each type of paper, and measurements were taken after 2 minutes of drying. Each dilution of blood was measured 15 times on each piece of paper and measured on the HemoCue 201+ to provide a reference hemoglobin measurement. Measurements were conducted in the following order: HemoCue, chromatography paper, HemoCue, nitrocellulose.

We also examined three speeds of nitrocellulose paper (HF090, HF135, and HF180) to determine the optimum speed for effective spreading and hemolysis of the blood. The nitrocellulose was dipped in 0.04 g/dL sodium deoxycholate in 1x PBS and allowed to dry. 10 μL of blood were spotted onto each speed of nitrocellulose. Visual inspection showed that HF090 gave the most consistent lysis of the RBCs (other speeds of nitrocellulose showed some plasma separation when blood was applied).

6.3.3. Clear tape increases length of time allowed from sample collection to measurement

We tested if covering the blood sample on nitrocellulose with clear tape (Scotch Magic tape, 3M, Maplewood, Minnesota) reduced sample drying, thus providing clinicians a longer window of time in which to read the sample and obtain accurate results. 10 μL blood samples were spotted onto treated nitrocellulose paper and then covered with tape or left uncovered. Optical transmission was measured on a Cary UV-VIS spectrometer every 1 min after spotting for a total of 60 minutes. Three blood dilutions were made, and each dilution
was measured a total of six times: on three pieces of nitrocellulose paper (HF090) for each condition (tape or no tape).

### 6.3.4. Manufacturing disposable cartridges

For further experiments, disposable cartridges (Figure 6-1b) were prepared as follows: Cut nitrocellulose using a laser cutter. The notched corner ensures that the nitrocellulose can only be inserted into the reusable sample holder one way. Dip nitrocellulose into 0.04 g/mL sodium deoxycholate in 1x PBS and allow to dry. Apply Scotch tape to removable backing and cut on laser cutter. Apply nitrocellulose to the left half of the tape cutout, leaving the backing covering the right half of the tape. Store in a desiccated container. The final cuvette is shown in Figure 6-1b.

### 6.3.5. Accuracy

To train the algorithm and assess accuracy of the method in the lab, 21 dilutions of 300 μL each were created by diluting normal volunteer blood with plasma to simulate anemia or concentrating donor blood by removing plasma to simulate higher hemoglobin concentrations. The protocol was approved by the Rice IRB. The dilutions were measured on an Act Diff 2 hematology analyzer (Beckman Coulter, Brea, CA) to obtain a reference hemoglobin concentration. 10 μL of blood were spotted onto treated nitrocellulose, covered with tape, and measured on 3 different HemoSpec devices at 60, 90, and 120 seconds after spotting (rotating which device was used first). The samples were measured on a Cary UV-VIS spectrometer at 3 minutes after spotting. Two replicates were measured for each sample. To assess accuracy, leave-one-out analysis was performed: data from 20 dilutions were used to create a training algorithm, which was applied to the data from the remaining dilution.
This process was repeated for all dilutions. The final training algorithm for the field study used data from all 21 dilutions.

Accuracy of the method was also assessed using blood from 98 patients from LBJ Hospital in Houston, Texas. The protocol was approved by the Rice, M.D. Anderson, and Harris Health IRBs. Patients aged 18 years or older were eligible for the study if they attended LBJ oncology clinics, their treating physician requested a hemoglobin concentration assessment, and they provided informed consent. Venous blood was drawn for the standard of care, and the hemoglobin measurement was obtained. Our work has shown considerable variability in the hemoglobin concentration of consecutive drops of blood obtained from a fingerprick. To mitigate the effect of these physiological variations on our experiment, we collected up to 8 drops (approximately 80 μL) of fingerprick blood into an EDTA-coated collection tube (BD 365974). This pooled fingerprick blood was mixed well with a pipette. 10 μL were measured using a HemoCue 201+, which served as the gold standard for the fingerprick blood because the fingerprick hemoglobin concentration can vary from that determined using venous blood. The HemoCue 201+ is CLIA-waived and has been shown to have high accuracy in the laboratory. 10 μL of blood were applied with a pipette to treated nitrocellulose paper, covered with tape, and placed in the HemoSpec reader for analysis. An additional 10 μL was used to take a second reading on the HemoSpec if there was sufficient blood volume in the sample. Sufficient blood for analysis was collected from 70 patients. 21 of these samples were excluded due to inadequate mixing. This left 49 patient samples for analysis, of which 34 samples had sufficient volume for 2 measurements, for a total of 83 measurements on the HemoSpec.
6.4. Results & Discussion

6.4.1. Nitrocellulose is more precise than chromatography paper

Table 6-1. Variation of spectroscopic measurements using chromatography paper and nitrocellulose paper measured on a Cary UV-VIS spectrometer (a) and a portable HemoSpec reader (b). Percent coefficient of variation (%CV) for the papers represents the variation of the OD difference between 532 nm and 656 nm over 15 measurements. %CV for HemoCue represents the variation of the hemoglobin measurement over 30 measurements. Hemoglobin concentration is the average of all measurements on the HemoCue.

<table>
<thead>
<tr>
<th></th>
<th>Hb (g/dL)</th>
<th>%CV HemoCue</th>
<th>%CV Chromatography</th>
<th>%CV Nitrocellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Spectrophotometer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.6</td>
<td>0.5%</td>
<td>6.0%</td>
<td>1.7%</td>
</tr>
<tr>
<td></td>
<td>10.3</td>
<td>0.8%</td>
<td>5.4%</td>
<td>1.9%</td>
</tr>
<tr>
<td></td>
<td>4.4</td>
<td>0.8%</td>
<td>7.9%</td>
<td>1.8%</td>
</tr>
<tr>
<td>b. Portable reader (HemoSpec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>0.5%</td>
<td>10.0%</td>
<td>2.5%</td>
</tr>
<tr>
<td></td>
<td>10.7</td>
<td>0.7%</td>
<td>7.3%</td>
<td>2.2%</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>0.6%</td>
<td>9.9%</td>
<td>2.0%</td>
</tr>
</tbody>
</table>
As shown in Table 6-1, nitrocellulose shows a smaller coefficient of variation in transmission measurements than chromatography paper across a range of hemoglobin concentrations and on both a benchtop spectrometer and the HemoSpec reader. Nitrocellulose has a %CV an average of 3.8 times smaller than that of chromatography paper. Though the variation shown by nitrocellulose is not as small as that shown by the commercial HemoCue, it represents a substantial improvement over that seen when using chromatography paper. We hypothesize that chromatography paper has a higher variability because the random fiber orientation of the paper is not repeatable from sample to sample, while nitrocellulose offers a smooth surface without fibers.
6.4.2. Clear tape increases length of time allowed from sample collection to measurement

Figure 6-2. Three blood samples (with high, medium, and low hemoglobin concentrations) were measured on nitrocellulose paper either uncovered (a) or covered with tape (b). Absorbance readings were made every minute after spotting the blood on the paper for a total of 60 minutes. Three measurements were taken of each blood sample in each condition. Bold lines represent the average of the three measurements; shaded areas represent ± 1 SD. The hemoglobin concentration was calculated using an algorithm derived from a training set on blood spots covered by tape and measured at 2 min after spotting. The black vertical line represents the average time at which the samples differ by >7% from their initial hemoglobin measurement.

Figure 6-2 shows the calculated hemoglobin concentration versus time for three dilutions of blood corresponding to a high, medium and low concentration of hemoglobin spotted on treated nitrocellulose and either left uncovered (a) or covered with tape (b). The hemoglobin concentration was calculated using the algorithm described below in Laboratory Accuracy. To be considered a CLIA-waived test, hemoglobin analyzers must be accurate to within ±7% of the reference concentration, so we evaluated how long the samples could be left before the calculated hemoglobin concentration would change by greater than 7% from
the initial reading. Without tape, the calculated hemoglobin concentration changes by more than 7% after an average of only 2 minutes (high = 3 minutes, medium = 2 minutes, low = 2 minutes). When covered with tape, the hemoglobin concentration changes by more than 7% after an average of 22 minutes (high = 18 minutes, medium = 14 minutes, low = 34 minutes). Note that when covered with tape, the calculated hemoglobin concentration decreases over time rather than increases.

Covering the paper with tape also appears to reduce the variability of measuring the same blood sample, especially with high and medium concentrations at times greater than 20 minutes.
6.4.3. Laboratory Accuracy

Figure 6-3. In-lab results using donor blood diluted with plasma to simulate anemia. An ActDiff 2 hematology analyzer was used as a gold standard. Two separate HemoSpec measurements were made for each of 21 blood samples. The device was trained using leave-one-out analysis. (a) Frequency (bars) and cumulative percent (line) of samples reaching the given deviation from the reference device. (b) Bland-Altman plot showing the average of the calculated and reference hemoglobin concentrations on the x-axis and the difference between the two on the y-axis. Dotted lines show ±7% deviation, the requirement to be certified as CLIA-waived in the United States.

To train the algorithm and assess the accuracy of the method in the laboratory, we created 21 samples by adding or removing plasma from normal donor blood. Each sample was measured on an Act Diff 2 hematology analyzer as a gold standard and measured on two cuvettes placed in a HemoSpec reader, where the red and green optical densities, background measurements, and nitrocellulose adjustment were recorded. To assess the hemoglobin concentration, the HemoSpec reader was trained using leave-one-out analysis on the adjusted difference between red and green optical densities. The final algorithm used for further experiments (calculated using all 21 samples) was:
\[ y = 6.7509x^{1.5822} \]

where \( y \) is the hemoglobin concentration in g/dL and \( x \) is the optical density difference.

Figure 6-3 shows the results of this leave-one-out analysis as compared to the reference analyzer. Figure 6-3a shows the frequency and cumulative percent of samples achieving a given deviation from the reference. 98% of samples were within ±1 g/dL of the reference hemoglobin concentration. Figure 6-3b shows the data on a Bland-Altman plot with comparison to the ±7% requirements to be CLIA-certified for hemoglobin in the U.S. Absolute accuracy is higher at lower hemoglobin concentrations, and only 3 of 42 measurements were outside the required ±7%.
6.4.4. Field Accuracy

Figure 6-4. Field results using blood from 83 patient samples (49 patients). A HemoCue 201+ was used as a gold standard. Parts (a,b) use the training algorithm from in-lab analysis; parts (c,d) show the results from the algorithm trained on the field data using leave-one-out analysis. (a,c) Frequency (bars) and cumulative percent (line) of samples reaching the given deviation from the reference device. (b,d) Bland-Altman plot showing the average of the calculated and reference hemoglobin concentrations on the x-axis and the difference between the two on the y-axis. Dotted lines show ±7% deviation.

Figure 6-4 shows the results of assessing the device in the field with patient samples. When using the training algorithm developed in the lab (Figure 6-4a,b), 81% of samples were within ±1 g/dL of the HemoCue 201+ reference standard, and 98% were within 1.5 g/dL.

The Bland-Altman plot (Figure 6-4b), however, clearly shows that the device has a bias (average = -0.2 g/dL) against the reference standard. Further experiments need to be done
to determine if this bias was due to a shift in either the HemoCue or HemoSpec since training or if there were interfering substances seen in patient samples (such as a very high white blood cell count or lipidemia) that were not present in the diluted donor blood used for training.

We also assessed training the field data using leave-one-out cross validation (Figure 6-4c,d). (If a given patient sample had two HemoSpec measurements made, both measurements were withheld from a round of leave-one-out cross validation.) This analysis corrects for the bias, and results in 89% of measurements within ±1 g/dL of the standard. Further, 81% of samples are within the ±7% required by CLIA.

The hemoglobin values in this data set as measured by the HemoCue ranged from 9.1 to 16.7 g/dL, a much narrower range than that assessed in the laboratory. The data in Figure 6-3 and in previous experiments suggest that the absolute accuracy of the device is higher at lower hemoglobin concentrations, and greater accuracy is needed at these severe anemia levels for clinical decision-making. The device should be assessed in a low-resource setting with a higher prevalence of moderate and severe anemia to more thoroughly understand the accuracy with patient samples at all anemia levels.

6.4.5. Cost Analysis

Using nitrocellulose improves the accuracy of the device without significantly increasing the cost of goods. A single cuvette of Whatman Chr4 paper bought from Sigma Aldrich is $0.006. The cost of goods to make a single cuvette of HF090 bought from EMD Millipore is $0.013. Scotch tape bought from Staples.com adds a further $0.003 per cuvette, leading to a final per-test cost of goods of approximately $0.016 per cuvette in volumes.
under 5,000. This low per-test cost would be expected to decrease further when the materials are purchased in bulk.

6.5. Conclusion

This paper has shown improvements in the disposable and portable reader for measuring hemoglobin concentration at the point of care. Nitrocellulose paper offers an average of 3.8 times higher precision than chromatography paper, which translates to higher accuracy. Covering the nitrocellulose with tape reduces drying speed and increases the time a clinician has to read the sample by 20 minutes. These improvements come with a very small per-test cost increase (from $0.006 with chromatography paper to $0.016 with nitrocellulose and tape at low volumes). The reader, cuvette, and sample holder were adjusted to ensure that the sample can only be inserted in one direction, and changes to the calibration procedure ensure that multiple HemoSpec devices report the same answer for a given sample.

In a laboratory setting, 76% of samples were within 0.5 g/dL of the reference, and 98% were within 1 g/dL, our clinical target. Only 3/42 samples were outside the ±7% requirements to be CLIA-waived. In the field, 89% of samples were within 1 g/dL of the reference standard. 81% of samples were within CLIA’s ±7% requirement, and 96% were within ±10%. Our 95% limits of agreement for the patient samples were -1.26 to +1.27 g/dL, which compare favorably to HemoCue’s 95% limits of agreement of -0.68 to + 2.25 g/dL when assessed in the field, although Gomez-Simon et al. were not controlling for drop-to-drop variability in fingerprick samples. Our low-cost method offers considerable improvement in accuracy over WHO’s Hemoglobin Color Scale (approximately $0.02/test),
which showed 95% limits of agreement of -3.50 to +3.11 g/dL in the laboratory and only 67% of measurements within 2 g/dL of a reference standard in the field \cite{36,38}.

Future work should evaluate the sources of the bias initially seen in the field. The device also needs to be evaluated in a low-resource setting and when operated by clinicians.

This new device and method are thus sufficiently low cost, easy to use, and accurate for detecting anemia at the point of care in low-resource settings.
Chapter 7

A Novel Point-of-Care Strip Test to Diagnose Sickle Cell Anemia

7.1. Abstract

Sickle cell disease (SCD) affects about 300,000 babies born each year, and 76% of these children are born in sub-Saharan Africa. Low-cost treatments are available for sickle cell disease, but there is no robust newborn screening program to identify patients in need of treatment. Additionally, emergency rooms in high-resource settings need a rapid diagnostic for SCD to differentiate disease sufferers from drug-seekers presenting with pain. We developed and evaluated a competitive lateral flow assay that identifies SCD in 15 minutes using undiluted whole blood. Blood was mixed with antibody-coated blue latex beads in a

This chapter was prepared with the assistance of Brady Hunt, Bailey Flynn, Petri Huhtinen, Russell Ware, and Rebecca Richards-Kortum and will be submitted for publication.
tube and applied to the strip. Strips were then placed in a well of running buffer and allowed to run for 10 minutes. The test can detect SCD and can detect sickle cell trait (SCT) with a blood composition of 50 – 5% HbA (50 – 95% HbS). We evaluated the test using 41 samples from patients with SCD, SCT, and normal blood. With visual inspection or quantitative analysis, we found a 63% accuracy for differentiating among SCD, SCT, and normal blood, but a 98% accuracy when differentiating SCD from normal and SCT samples as a group (90% sensitivity and 100% specificity for identifying SCD). A higher affinity antibody to sickle hemoglobin (HbS) might enable better differentiation between SCT and normal blood, and the test needs to be evaluated in field settings with high and low prevalence of SCD. This test represents a promising method for rapidly identifying SCD patients as newborns or upon presentation to the emergency room.

7.2. Introduction

A recessively inherited mutation of the hemoglobin gene causes sickle cell disease (SCD); instead of normal adult hemoglobin (HbA), patients with SCD produce sickle hemoglobin (HbS). Symptoms of SCD include severe anemia, pain, and decreased immune function\(^\text{10}\). Homozygotes for the sickle cell gene mutation who produce only HbS (genotype SS) are affected by the disease. Heterozygotes, producing both HbA and HbS (genotype AS), have sickle cell trait (SCT) and suffer few symptoms of the disease, though they can pass on the gene to their children.

Piel et al. estimated that 312,302 babies were born with sickle cell disease in 2010 and a further 5,476,407 were born with sickle cell trait\(^\text{11}\). An estimated 75.5% of babies with sickle cell disease are born in sub-Saharan Africa, where there are few resources to diagnose
and treat the disease. Global distribution of the sickle cell mutation follows the historic distribution of malaria because persons with SCT have some protection against dying from malaria.

High-resource settings use organized newborn screening to identify SCD patients quickly. Once identified, patients have access to effective treatments to reduce painful sickle cell crises, increase hemoglobin concentration, and prevent infection, and 90-99% of children with SCD survive to the age of five years. However, lifespans for people with sickle cell disease in the U.S. are still an average 3 decades shorter than the general population. The challenge for patients in high-resource settings is managing vaso-occlusive crises. The current recommendation is to treat patients rapidly with opioid pain relievers.

However, when presenting at the emergency room, these patients can easily be mistaken as drug-seeking or drug-addicted patients. A rapid diagnostic appropriate for use in an emergency room would help distinguish sickle cell patients in true need from drug seekers or drug-addicted patients, especially with the recent increased scrutiny of opioid use in the U.S. Additionally, an autopsy study of causes of death in U.S. sickle cell patients suggests that a large percentage of deaths (27.7%) occurred in the first 24 hours after presentation of the patient to the hospital, so rapid identification and treatment during these crises is essential.

Low-resource settings lack robust newborn screening programs for SCD. Only an estimated 20-50% of affected children in Africa survive to the age of five, typically succumbing to infections due to the damage caused by sickled red blood cells to the spleen. Treatments such as hydroxyurea (to increase the production of fetal hemoglobin, HbF) and antibiotics (to prevent infection) are available at low cost in these areas, but clinicians do not
have a simple, low-cost method to identify SCD patients in need of therapy. There is a need for a point-of-care diagnostic appropriate for newborn screening in a low-resource setting.

Isoelectric focusing (IEF) and high performance liquid chromatography (HPLC) are considered gold standards for sickle cell diagnosis\textsuperscript{16}. IEF separates proteins in a pH-gradient gel based on slight differences in isoelectric point, and HPLC separates proteins based on differing interaction times with a column. While these diagnostic methods are sensitive and specific, both require expensive equipment and extensive training and are thus not appropriate for point-of-care use, especially in low resource settings.

To meet this need, several tests have been developed to screen for SCD at the point of care. Tests like SickleDex (Streck, Omaha, NE) exploit solubility differences in the HbA and HbS molecules. Whole blood is added to a liquid containing reagents to lyse red blood cells, deoxygenate the hemoglobin, and induce precipitation of the HbS molecule\textsuperscript{17}. If present, HbS precipitates and makes the solution turbid, while solutions lacking HbS remain clear. SickleDex is simple to use and sensitive for the presence of HbS, but it is not specific. Erroneous results can be caused by sickle cell trait, severe anemia, blood from neonates with high concentrations of fetal hemoglobin (HbF), and other factors, which can all be expected in a newborn screening setting in a region with high prevalence\textsuperscript{17}. Yang et al. have adapted this principle to a paper-based assay, where blood is mixed with SickleDex reagents and spotted on paper\textsuperscript{44}. Precipitated HbS is trapped in the fibers of the paper, while free HbA wicks away from the center of the spot. The color intensity of the outer ring can be used to differentiate between patients with SCD, SCT, and normal blood. In the United States, the method detected SCD with 93% sensitivity and 94% specificity using visual evaluation, and detected SCT in Cabinda, Angola with 94% sensitivity and 97% specificity\textsuperscript{45}. 
Lateral flow may be appropriate as a rapid, low-cost platform to diagnose SCD. Monoclonal antibodies specific to HbA, HbS, and even HbF or HbC (another common disease-causing hemoglobin mutation) are available and can be used to develop immunochromatographic assays to distinguish conditions such as hemoglobin SC disease and SCT that have a similar %S content and thus be indistinguishable by solubility methods. Recent studies examined the performance of SickleSCAN, a lateral flow assay to detect HbA, HbS, and HbC. In this test, polyclonal antibodies against HbS, HbC, and HbA are dried on the strip and used to capture hemoglobin molecules. A labeled antibody against the hemoglobin α subunit (common to all three hemoglobin variants) provides visual readout. To operate the test, 5 μL of blood is collected from the patient and then mixed with a lysis and diluent buffer. Several drops of this mixture are applied to the lateral flow strip, and blue lines form via a sandwich immunoassay to indicate the types of hemoglobin present in the sample. McGann et al. report a high sensitivity (98.3-99.5%) and specificity (92.5-94%) for the device to detect the presence of HbS and HbA, and 98.4% sensitivity and 98.6% specificity for detecting SCD using 139 venous blood samples in the laboratory relative to a gold standard of capillary zone electrophoresis. However, SickleSCAN requires a precise measurement of blood volume, and the user must dilute the blood before adding it to the device. A lateral flow strip that could accept unmeasured blood directly from a fingerprick would reduce cost and substantially simplify test operation.

In this paper, we present a point-of-care lateral flow test to distinguish among SCD, SCT, and normal blood. This test uses a competitive lateral flow format which is less sensitive to saturation than the sandwich assay format and does not require the user to meter blood volume or dilute the sample.
7.3. Methods

Conventional sandwich immunoassays are ideal for detecting antigens present at relatively low concentrations, from approximately 1 ng/mL to 500 μg/mL, though these ranges vary widely based on antibody concentration and antigen choice⁴⁶,⁴⁷. When the antigen is present at very high concentration, it can fill all available binding sites on both the capture antibody and the detection antibody, preventing a sandwich from forming and thus giving no visible capture line (the “hook effect” or “prozone effect”)⁴⁸. Hemoglobin is present in a drop of blood at a concentration of approximately 4 g/dL to 20 g/dL, thus a sandwich assay detecting hemoglobin requires 40-400-fold dilution to avoid the hook effect. The SickleSCAN sandwich assay uses 200-fold dilution. An alternative is a competitive lateral flow assay in which antigen dried at the capture line competes with antigen in the sample to bind to a labeled detection antibody. In the absence of antigen in the sample, a signal appears at the capture line, whereas when antigen is present, the capture line has no signal.
Figure 7-1. (a) Schematic of lateral flow test strip for three possible conditions: normal, sickle cell trait, and sickle cell disease blood. HbA, HbS, and an anti-mouse control antibody are dried on the strip at the capture and control lines. Two populations of latex beads (one conjugated to anti-HbA, the other to anti-HbS) and the blood sample are flowed up the strip. (b) Resulting visible readout on the strip for normal, sickle cell trait, and sickle cell disease blood. (c) Scanned images of example strips run with patient blood. Top image shows full-color scan; bottom image shows red channel of same image. Labels on the side are a guide to interpreting the competitive assay: a line present at the “A” line indicates HbAA, at the “S” line indicates HbSS, and at neither indicates HbAS. The positive control line must be present for the test to be valid.
Figure 7-1 shows a schematic of the competitive assay developed to detect SCD. HbS and HbA are dried on the strip (“capture hemoglobins”), and an anti-mouse antibody serves as a positive control. Sample blood is mixed in a tube with two populations of blue latex beads: one conjugated to mouse anti-HbA antibodies and the other to mouse anti-HbS antibodies. Blue latex was chosen to provide a strong contrast against a background of red hemoglobin. When the sample and latex bead mixture is applied to the strip and the strip is placed in running buffer, the beads flow down the strip and bind to the capture proteins if no antigen is present. If the antigen is present, the beads cannot bind to the capture protein. For example, if HbAA blood is applied, the sample HbA blocks the binding sites of the anti-HbA latex, preventing it from binding to the capture HbA. The anti-HbS latex is free to bind to the capture HbS, and either type of latex may bind to the positive control line, resulting in two visible lines.

7.3.1. Preparation of Lateral Flow Strips

Hemoglobin was extracted from leftover clinical blood samples under a protocol approved by Rice University’s and Cincinnati Children’s Hospital’s IRBs to serve as capture hemoglobin on the strips according to the following protocol. 1 mL PBS was added to 100 μL of whole blood to wash the red blood cells. The solution was spun for 2 min at 1,500 g and the supernatant was discarded. 400 μL of RIPA buffer (product number 89900, Thermo Scientific, Waltham, MA) with added protease and phosphatase inhibitors (product number 78440, Thermo Scientific) was mixed with the pellet to lyse the red blood cells. The tube was then spun at 13,400 g for 15 minutes, and the supernatant was collected. The protein present in the supernatant was quantified with a BCA Protein Assay (product number 23225, Thermo Scientific). Aliquots were stored at -80°C.
Before application to the lateral flow strip, the hemoglobin was thawed and diluted in PBS to 1 mg/mL. Rabbit anti-mouse IgG (SC-358919, Santa Cruz Biotechnology, Dallas, TX) was spotted at the positive control line. Proteins were applied to a nitrocellulose membrane card (HF135, Merck KGaA, Darmstadt, Germany) using a lateral flow reagentstriper (Claremont Bio, Upland, CA) set at 4.5V with a flow rate of 0.15 mL/min. Strips were allowed to dry for 24 hours at room temperature. A cellulose absorbent pad (17 mm x 300 mm, Merck KGaA) and glass fiber pad (10 mm x 300 mm, Merck KGaA) were applied to the card, each overlapping the nitrocellulose by approximately 2 mm. The absorbent pad was covered with laboratory tape in order to ensure secure it to the nitrocellulose membrane. Strips were then cut to a width of 3.5 mm using a guillotine cutter (Index Cutter II, A-Point Technologies, Gibbstown, NJ). Strips were stored in tubes with desiccant for up to one month before use.

7.3.2. Preparation of Latex Conjugate

Latex conjugation kits (400 nm, blue) were obtained from Innova Biosciences (Cambridge, UK). Monoclonal anti-HbS and anti-HbA antibodies raised in mice were acquired from PerkinElmer (Waltham, MA). The antibodies were first exchanged into a new buffer using the Innova Antibody Concentration and Clean Up Kit into buffer A (anti-HbS antibody) or buffer B (anti-HbA antibody). Antibodies were covalently conjugated to the latex beads according to kit directions, with anti-HbA added at a concentration of 0.4 mg/mL instead of the recommended 0.1 mg/mL. The conjugate was stored in the provided resuspension buffer with 0.1% BSA.

Strips were run according to the following basic procedure: the two populations of latex beads were mixed with a sample of blood in a tube and allowed to incubate at room
temperature for approximately 5 minutes. The mixture was spotted onto the glass fiber pad of the lateral flow strip, which was placed upright in a 96-well plate containing 40 μL of PBS + 0.5% Tween-20. The strip was allowed to run for 10 minutes, and it was then removed and scanned using a flatbed scanner.

7.3.3. Quantification

We developed an automated method to interpret the strips. The strips were scanned using a flatbed scanner, and the full color images were manually cropped using ImageJ. The red channel values were extracted to reduce the signal associated with the red blood cells. The red channel values were averaged horizontally across the strip to obtain a 1-dimensional intensity profile of the strip from bottom to top. The center of the positive control line was defined to be the intensity minimum furthest along the strip. The A and S lines were located based on a fixed pixel offset from the positive control line. Background regions were defined for each line as a 30-pixel high region centered 60 pixels below the given line. Signal-to-background ratio (SBR) was calculated for each line and compared to the threshold set in the following section. The presence of signal at either of the two capture lines (HbA or HbS) determines whether the sample contained HbS, HbA, or both, and is thus consistent with either SCD, SCT, or normal blood.

7.3.4. Determining a Signal-to-Background Ratio Threshold

We determined the SBR threshold for each line by measuring the SBR of various volumes of blood added to the strip for each type of hemoglobin. 1.75 μL of anti-HbS latex conjugate, 5.25 μL of anti-HbA latex conjugate, and various volumes of blood (0 – 87.5 μL) were mixed in a tube. Aliquots corresponding to a blood volume of 0, 0.5, 1, 2, 3, 5, 10, 15,
and 25 μL of blood were added to three separate strips. Blood samples were either HbAA (16.2 g/dL) or HbSS (84.5% HbS, 9% HbF, 9.6 g/dL). HbAA blood samples were obtained from normal volunteers under a protocol approved by the Rice University IRB, and HbSS blood samples were obtained from leftover clinical samples under a protocol approved by the Rice University and Cincinnati Children’s Hospital IRBs. The strips were scanned and analyzed visually and with the quantification algorithm. The signal-to-background ratios for the negative lines (A lines on the strips assessed using HbSS blood, volumes 0.5 – 10 μL, and S lines on the strips assessed using HbAA blood, volumes 0.5 – 5 μL) were used to set a signal-to-background threshold for further experiments.

### 7.3.5. Ratio of HbA: HbS

We assessed strip performance for a range of ratios of HbA: HbS. To have a hemoglobin concentration matching that of the HbSS blood, HbAA blood was diluted with AB+ plasma obtained from the Gulf Coast Regional Blood Bank under a protocol approved by the Rice University IRB. HbSS and diluted HbAA blood of compatible blood types were mixed to simulate HbAS samples at various ratios of hemoglobin. 1.75 μL of anti-HbS latex conjugate, 5.25 μL of anti-HbA latex conjugate, and 10.5 μL of blood were mixed in a tube. Aliquots corresponding to a blood volume of 3 μL of blood were added to three separate strips. The strips were scanned and analyzed.

### 7.3.6. Assessing Accuracy with Patient Samples

Finally, we assessed the accuracy of the strips at diagnosing patient samples. Forty-one patient samples were acquired from leftover clinical samples under a protocol approved by the Rice University and Cincinnati Children’s Hospital IRBs. High performance liquid
chromatography (HPLC) was used as the gold standard diagnosis, and samples were analyzed by lateral flow within 3-9 days of collection. Hemoglobin concentration was assessed using a hematology analyzer (Ac·T diff2; Beckman Coulter, Brea, CA).

3 μL of each sample were mixed with 0.5 μL anti-HbS latex conjugate and 1.5 μL anti-HbA latex conjugate in a tube and incubated at room temperature for 5 minutes. The strips were run and scanned. A first reader evaluated the diagnosis within 5 minutes of scanning the strip. Two additional readers evaluated the diagnosis by viewing scanned images of the strips. Finally, the scanned images were assessed quantitatively. The lateral flow operator and all readers were blinded to the gold standard diagnosis.
7.4. Results

7.4.1. Determining a Signal-to-Background Ratio Threshold

Figure 7.2. (a) Representative strips run with varying amounts of HbAA blood. Top image shows full-color scan; bottom image shows red channel of the same image. (b) Signal-to-background ratio of strips in part a. Error bars represent ±1 SD of three strips. *10 μL represents the average of only 2 strips. (c) Representative strips run with varying amounts of HbSS blood. (d) Signal-to-background ratio of strips in part c.

Figure 7.2a shows representative images of strips run with varying volumes of HbAA blood. At volumes ≥10 μL for HbAA blood and ≥15 μL for HbSS blood, a positive control line was not consistently present within the 10 minute assay run time, suggesting that
the test is only appropriate for volumes from 0.5 μL to 10 μL. Figure 7-2b quantifies the signal-to-background ratio (SBR) at each test line over this range of volumes. The SBR of the S line in Figure 7-2b with 0.5 – 5 μL of blood (or 81 – 810 μg of HbA) was used to generate an SBR threshold of 1.06 (average SBR + 3*SD) for the S line in further experiments. At 10 μL, only 2 of the 3 strips produced a visible positive control line, so this volume was not included in the SBR threshold.

Similarly, Figure 7-2c shows representative images of strips with varying volumes of HbSS blood. Figure 7-2d quantifies the SBR at each test line, and the SBR of the A line on strips with visible control lines (from 0.5 – 10 μL, or 41-811 μg of HbS) generates a threshold of 1.13 for the A line for subsequent experiments.

**Ratio of HbA: HbS**

![Image](image.png)

Figure 7-3. (a) Representative strips run with varying ratios of HbA: HbS. Top image shows full-color scan; bottom image shows red channel of the same image. (b) Quantification of strips in part a. Dotted lines represent the thresholds determined in Figure 3. Error bars represent ±1 SD.
Figure 7-3 examines the effects of varying the ratio of HbA to HbS at a volume of 3 μL of blood. At 0% HbA (100% HbS), only the S line is above the threshold, and the strips are classified as SCD. From 5-50% HbA (95-50% HbS), both the A and S lines are below their respective thresholds, and the strips are classified as SCT. From 60-100% HbA (40-0% HbS), the A line is above the threshold, and the strips are classified as normal.

7.4.2. Assessing Accuracy with Patient Samples

The patient samples were interpreted with three methods: live visual interpretation immediately after the strips were run (1 reader), visual interpretation using scans of the strips (2 readers), and automated analysis. The hemoglobin concentration of samples ranged from 4.6 – 16 g/dL (median of 11 g/dL).
Results from visual interpretation are shown in Table 7-1. (Results for each sample, including HPLC hemoglobin percentages and the hemoglobin concentration of each sample, can be found in Table B 1, Appendix.) The results of the experiment assessing the ratio of HbA: HbS show that our test is not capable of distinguishing normal from SCT samples at

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high amounts of HbA. Therefore, we first assessed the ability to distinguish SCD from SCT and normal samples treated as a group using patient samples (Table 7-1a). Table 7-1a represents the results from all 3 readers, who were concordant. By grouping normal and SCT samples, we achieved an accuracy for visual interpretation of 98%, with 90% sensitivity and 100% specificity for identifying SCD. When identifying SCT separately, reading the strips in person (Table 7-1a) resulted in an overall accuracy of 49%. 20 of the 21 SCT (HbAS) samples were incorrectly interpreted as AA. The two readers viewing scans of the strips (Table 7-1c), however, were able to correctly identify 7 of the 21 SCT strips for an overall accuracy of 63%.

**Table 7-2.** Confusion matrices comparing gold standard diagnosis (HPLC) to quantitative analysis. (a) Interpretation based on grouping AA and AS together. (b) Interpretation based on three categories of AA, AS, and SS. One strip was classified as having both A and S lines present, which does not correspond to a diagnosis.

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|                | Strip Test |            |            |            |            |            |
| (b)            | AA         | AS         | SS         |            |            |            |
| HPLC           | AA         | 10         | 0          | 0          |            |            |
|                | AS         | 14         | 7          | 0          |            |            |
|                | SS         | 0          | 0          | 9          |            |            |
|                |            | **24**     | 7          | 9          |            |            |
The results of assessing the strips quantitatively using the analysis program and the SBR threshold established in Figure 7-2 are shown in Table 7-2. One of the strips was interpreted as invalid when both the A and S lines were above the threshold and does not appear in these tables. When normal and SCT diagnoses are grouped, automated analysis achieves 98% accuracy with 90% sensitivity and 100% specificity for identifying SCD (Table 7-2a). Separating the strips by three possible diagnoses (Table 7-2b) gives very similar results to visual interpretation of scanned strips (63% accuracy).

7.5. Discussion/Conclusions

We developed a competitive lateral flow test capable of identifying SCD in patient samples with 90% sensitivity and 100% specificity with either visual or automated interpretation. The test uses 3 μL of undiluted whole blood and requires only 10 minutes to run.

We assessed our strip with patient samples of varying hemoglobin concentration and composition. 2 of 3 readers and the quantitative analysis were able to correctly classify the sample with the lowest hemoglobin concentration (4.6 g/dL) as SCT, and all methods correctly identified the sample with the highest hemoglobin concentration (16.0 g/dL) as normal. All methods correctly interpreted the SCD strip with the highest percentage of HbF (32.9%).

SCT can be detected at 50 – 5% HbA (50 – 95% HbS) as the absence of both test lines. However, clinical specimens of SCT contain 24-45% HbS49,50. At these percentages, our test appears to give a diagnosis of normal, so we cannot reliably distinguish SCT from
normal samples. A higher affinity anti-HbS antibody could potentially shift our ability to
detect SCT into the clinical range.

This test can distinguish SCD from either SCT or normal blood, so the test could be
used to identify patients who need treatment (SCD) from those who do not (SCT and
normal).

Further research needs to be done to more fully examine the effects of varying
hemoglobin concentration or the percent of non-detectable hemoglobins, such as fetal
hemoglobin (HbF) or other mutations like HbC. We would expect a wide range of
hemoglobin concentrations when testing healthy newborns and anemic adults affected by
SCD, and similarly the percent of HbF can vary from roughly 0% to 80% when screening
adults and newborns\textsuperscript{51,52}. These additional factors may affect the amount of blood necessary
to obtain a clear result using our test.

Another limitation of our test is that the latex beads and sample blood must be
mixed in a tube before application to the strip. The latex should be dried into a conjugate
pad before testing the device in the field. The accuracy of the strip should be assessed in
areas of low- and high-prevalence for SCD. Given sensitive and specific antibodies, the
design of this strip could be expanded to detect other hemoglobin variants such as HbF and
HbC.

This competitive assay shows promise for identifying SCD (90% sensitivity, 100%
specificity), but needs improvement to identify SCT samples and to be used in the field.
Chapter 8

Conclusions

This thesis presents an analysis of the drop-to-drop variation of blood components in fingerprick blood and describes the development of new diagnostic tools to assess hemoglobin concentration and diagnose sickle cell anemia.

8.1. Drop-to-Drop Variation of Cellular Components of Fingerprick Blood

This study (Chapter 3) assessed the variation in hemoglobin, platelet count, and white blood cell count and differential in successive drops of fingerprick blood. It is important for clinicians to understand this degree of variability when interpreting results obtained using fingerprick blood, and it is important to account for this variation when assessing the accuracy of new point-of-care devices using fingerprick blood. The average percent coefficient of variation (CV) for successive drops of fingerprick blood was higher by up to 3.4 times for hemoglobin, 5.7 times for WBC count, 3 times for lymphocyte count, 7.7
times for granulocyte count, and 4 times for platelets than in venous controls measured using a hematology analyzer. The average percent CV for fingerprick blood was up to 5 times higher for hemoglobin than venous blood measured using a point-of-care hemoglobinometer. Fluctuations in blood parameters with increasing volume of fingerprick blood are within instrument variability for volumes equal to or greater than 60 to 100 μL.

These data suggest caution in using the results of fingerprick hemoglobin and WBC tests for clinical decision making. The options for clinicians seem to be to (1) accept the inherent variability of fingerprick blood as a trade-off for ease of blood collection; (2) collect, read, and average multiple fingerprick samples, gaining accuracy but sacrificing cost and time; or (3) collect and analyze venous blood.

To assess the accuracy of point-of-care devices using fingerprick blood, researchers should collect multiple drops of blood in an anti-coagulant-coated tube, mix the blood thoroughly, and then apportion the blood to a standard device and the device under investigation to avoid under-estimating accuracy.

Future work should assess variability in fingerprick blood components with a larger number of sample donors to better quantify variation. For example, the hemoglobin concentration of donor F varied by only 0.5 g/dL over 6 drops, while that of donor E varied by 2.5 g/dL, and with a small number of donors it is difficult to determine which degree of variability is more common.

Additionally, similar studies should be performed to study the drop-to-drop variability in a range of analytes commonly assessed using fingerprick blood, including
glucose and bilirubin. The greatest challenge in assessing analytes seems to be finding an accurate gold standard that is capable of measuring tiny drops of fingerprick blood.

8.2. Measurement of Hemoglobin Concentration

The studies in Chapters 4-6 describe the development and evaluation of a low-cost, point-of-care method to assess blood hemoglobin concentration.

To measure hemoglobin concentration, blood is spotted on paper treated with sodium deoxycholate to lyse the red blood cells. Transmission measurements are made of the paper at a green wavelength, where hemoglobin absorbs strongly, and at a red wavelength, where hemoglobin does not absorb strongly. The difference in transmission at the two wavelengths corresponds to the hemoglobin concentration. Lysing the red blood cells yields a more even distribution of hemoglobin across the paper and results in a higher accuracy than unlysed blood. The wavelengths 528 nm and 656 nm were chosen to give a stable reading over time. The calculated hemoglobin concentration does not change when using blood drops of 5 – 25 μL, which means the method can be used with unmetered drops of blood at the point of care.

Initial training and validation of the device using chromatography paper and venous blood from hospitalized patients showed 95% of samples were within 2 g/dL of a HemoCue 201+ analyzer (Chapter 4). The design of the reader was then improved to use laser modules as light sources, a stable optical mount, and a simple calibration process before evaluating the device using fingerprick samples in Blantyre, Malawi. Here, 86% of samples were within 2 g/dL of a HemoCue 201+ standard (Chapter 5).
Finally, the disposable was changed from chromatography paper to nitrocellulose paper to facilitate greater repeatability and thus greater accuracy. The percent coefficient of variation for measurements made on nitrocellulose was an average 3.8 times smaller than those made on chromatography paper. The sample was covered with clear tape to increase the length of time allowed from sample collection to measurement from approximately 2 minutes to 22 minutes. In the laboratory, these changes resulted in 100% of samples within 2 g/dL of a reference standard, and 98% of samples were within ±1 g/dL of the standard. The device was then evaluated using fingerprick blood from 49 oncology patients in Houston, TX, resulting in 89% of samples within ±1 g/dL of the reference standard. Further experiments need to be done to determine the source of the bias seen when measuring patient blood.

The final per-test cost of this method is approximately $0.016 (cost of goods), representing a significant reduction from the $1.00 per test (retail price) of HemoCue cuvettes in Malawi. With a low per-test cost and high accuracy, the method seems appropriate for diagnosing anemia in low-resource settings.

Future work should evaluate the method with more patients in low-resource settings and assess the accuracy when operated by minimally-trained users. Special attention should be paid to samples known to interfere with other methods, such as samples with high white blood cell count, lipidemia, or high bilirubin concentration.

8.3. Diagnosis of Sickle Cell Anemia

The study in Chapter 7 describes the development and evaluation of a competitive lateral flow assay that identifies SCD in 15 minutes using undiluted whole blood. Blood is
mixed with antibody-coated blue latex beads in a tube and applied to the strip, which is added to running buffer and allowed to run for 10 minutes. The test can identify sickle cell trait (SCT) with a blood composition of 50 – 5% HbA (50 – 95% HbS). Test performance was evaluated using 41 samples from patients with SCD, SCT, and normal blood. With visual inspection or quantitative analysis, the test showed a 63% accuracy for differentiating among SCD, SCT, and normal blood, but a 98% accuracy when normal and SCT samples were grouped together (90% sensitivity and 100% specificity for identifying SCD).

It may be possible to adjust some components on the strip (such as capture protein concentration and the amount of anti-HbS antibody per bead) to enable better sensitivity to HbS and thus detect SCT more reliably in the clinical range. However, developing a more sensitive anti-HbS antibody is a better long-term solution, especially if the test is to be expanded to also detect variants like HbF and HbC.

The test needs to be thoroughly evaluated with blood of different volumes, different hemoglobin concentrations, different ratios of HbA:HbS, and the presence and concentration of hemoglobin variants not currently detected by the strip, like HbF and HbC. Future work should prepare the strip for field evaluation by drying the latex beads into a conjugate pad and evaluating the feasibility of adding blood directly from a fingerprick to the strip. Finally, the strip needs to be evaluated in the full range of end-use settings: low-resource newborn screenings, high-resource emergency rooms, and in areas of low and high prevalence for SCD.

In conclusion, this thesis achieved three specific aims:
Specific Aim 1: Designed and built a low-cost (<$0.02 per test), point-of-care method for measuring hemoglobin concentration using blood spotted on nitrocellulose paper, lysed by sodium deoxycholate, covered with clear tape, and measured spectroscopically using a custom-build reader.

Specific Aim 2: Evaluated the accuracy of the hemoglobin method in the field in both Blantyre, Malawi and Houston, TX, achieving 89% of samples within ±1 g/dL of the reference standard.

Specific Aim 3: Designed, built, and tested a low-cost, point-of-care method for determining sickle cell status capable of identifying SCD with 90% sensitivity and 100% specificity.
References


29. Boulton F, Nightingale M, Reynolds W. Improved strategy for screening prospective


45. Piety NZ, Yang X, Kanter J, Vignes SM, George A, Shevkoplyas SS. Validation of a


Appendix A

Table A 1. Average, standard deviation, percent CV, and range of 6 drops of fingerprick blood measured on a hematology analyzer for each donor. LY = lymphocyte count, GR = granulocyte count, MO = monocyte count.

<table>
<thead>
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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
</tr>
</thead>
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<tr>
<td>Hb (g/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>11.2</td>
<td>13.4</td>
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<td>14.0</td>
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<td>1.4%</td>
<td>4.3%</td>
<td>3.3%</td>
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<td>1.5</td>
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<td></td>
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<td>4.6%</td>
<td>3.5%</td>
<td>3.7%</td>
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<td>1.6</td>
<td>2.9</td>
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<td>LY (x10⁶ cells/μL)</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>1.9</td>
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<td>0.10</td>
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<td>6.7%</td>
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<tr>
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<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
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<td>J</td>
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<tr>
<td>GR</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x10^6</td>
<td>A</td>
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<td>3.1</td>
<td>3.3</td>
<td>5.9</td>
<td>7.7</td>
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<tr>
<td>cells/μL</td>
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<td>0.32</td>
<td>1.00</td>
<td>0.20</td>
<td>0.61</td>
<td>0.23</td>
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</tr>
<tr>
<td></td>
<td>(%)</td>
<td>CV</td>
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<td>5.4%</td>
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</tr>
<tr>
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<td>/μL</td>
<td>(%)</td>
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Table A 2. Average, standard deviation, percent CV, and range of 10 drops of fingerprick blood measured on a point of care hemoglobinometer.

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<th>3</th>
<th>4</th>
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<td>4.2%</td>
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<td></td>
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<td>2.1</td>
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<td>1.7</td>
<td>1.9</td>
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### Appendix B

Table B 1. Patient sample interpretations by visual readers (Readers 1-3), quantitative analysis, and the gold standard diagnosis determined by HPLC with percents of HbA, HbF, and HbS. Hemoglobin concentration was measured with a hematology analyzer.

<table>
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<th>Strip #</th>
<th>Reader 1</th>
<th>Reader 2</th>
<th>Reader 3</th>
<th>Quantitative Analysis</th>
<th>HPLC</th>
<th>%HbA by HPLC</th>
<th>%HbF by HPLC</th>
<th>%HbS by HPLC</th>
<th>[Hb] (g/dL)</th>
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<td>SS</td>
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