**TITLE:**

**A Precision Medicine Tool for Measurement and Monitoring of Hemoglobin S in Sickle Cell Disease Patients Receiving Transfusion Therapy**

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**SHORT ABSTRACT:**

We have developed a point-of-care immunoassay to rapidly quantify hemoglobin S (HbS) levels during transfusion therapy for sickle cell disease (SCD) patients. By applying a small amount of treated blood to the device, the healthcare provider can determine the %HbS in a SCD patient to immediately inform clinical decisions.

**LONG ABSTRACT:**

Sickle cell disease (SCD) causes many severe health complications, including anemia, stroke, and acute chest syndrome. Red blood cell transfusion is the most commonly used therapy to treat or prevent these devastating complications. Other therapies include hydroxyurea therapy as well as bone marrow transplantation. Chronic intermittent transfusion is especially indicated to prevent recurrent strokes. However, transfusion therapy is associated with significant adverse effects (e.g., alloimmunization and iron overload). The point-of-care (POC) lateral flow immunoassay used here quantifies the %HbS in 15 min using a small patient blood sample. Utilizing this information, the healthcare provider is able to adjust blood transfusion volume for SCD patients to achieve the desired target HbS (most often < 30%), while reducing the risk of transfusion-related complications. When compared to laboratory hemoglobin electrophoresis data for 38 whole blood samples, the POC test performed with very high correlation and linear fit (slope = 0.9949, R2 = 0.9751). The strong agreement between the two hemoglobin S percentage (%HbS) quantifying methods shows that 89.5% of samples fall within ±5.2% HbS bias. The calibration for quantifying %HbS is built into the device to allow for an automated quantification of %HbS. This time- and cost-effective POC test thus allows the healthcare provider to make timely informed decisions when treating SCD patients, using accurate and updated data.

**INTRODUCTION:**

Sickle cell disease (SCD) is a hereditary blood disorder characterized by hemolytic anemia due to the propensity of the mutant hemoglobin to polymerize when deoxygenated, leading to deformation and ultimately hemolysis of red blood cells (RBCs).[1](#_ENREF_1) Approximately two out of every 1000 births worldwide and more than 10 of every 1000 births in Africa are affected by SCD.[2](#_ENREF_2) SCD is characterized by the presence of hemoglobin S (HbS), a structural variant of normal adult hemoglobin, and occurs when mutated versions of the hemoglobin gene are inherited from both parents.[3](#_ENREF_3) Inheriting the HbS gene results in production of abnormal beta globin chains that polymerize when deoxygenated. The polymerization results in sickle shaped RBCs that have a markedly shortened life span, leading to moderate to severe anemia. Other effects of this process and the RBC abnormalities it engenders include increased RBC adhesion, activation of leukocytes and platelets, oxidative damage, and activation of coagulation and inflammatory pathways[4](#_ENREF_4), all of which contribute to vaso-occlusion, as well as to complications such as strokes and acute chest syndrome.[3](#_ENREF_3)

The severity of SCD varies greatly amongst individuals and correlates with a number of both hematologic and non-hematologic factors.[5](#_ENREF_5),[6](#_ENREF_6) The rate of stroke and other complications in high-risk patients could effectively be reduced by more than 80% through the implementation of transfusion therapy.[7](#_ENREF_7) Chronic RBC transfusion limits the rate of stroke and consequently improves the life of SCD patients, but alloimmunization and severe iron overload have severe adverse effects[7-10](#_ENREF_7). Implementation of this valuable therapy appropriately and judiciously is therefore critical in prevention of both stroke and reducible complications. The goal of chronic RBC transfusion for patients with SCD is to: (i) increase the [Hb] (to 9-10 g/dL) to improve the oxygen-carrying capacity of blood; (ii) dilute sickle Hb (to HbS <30%) to decrease the multiple downstream effects listed above that contribute to stroke and vaso-occlusion; and (iii) increase tissue oxygenation to suppress the production of hemoglobin polymers.[7](#_ENREF_7),[8](#_ENREF_8)

Determining the need for transfusion therapy and the appropriate volume to transfuse for SCD patients is largely based on the pretransfusion Hb level, the pretransfusion %HbS, body weight, and clinical condition. Common methods used for monitoring the efficiency of transfusion therapy are Hb electrophoresis[11](#_ENREF_11), high performance liquid chromatography[12](#_ENREF_12), or isoelectric focusing[13](#_ENREF_13),[14](#_ENREF_14). These tests are performed at a high cost with long processing times. Thus, determining a SCD patient’s need for transfusion therapy and the appropriate volume to transfuse is still largely based on the pretransfusion Hb level, body weight, and previous quantitative HbS measurements. Basing these decisions instead on the current %HbS could help tailor chronic transfusion for stroke prophylaxis, as well as acute transfusion for other SCD complications, more directly and effectively[15](#_ENREF_15).

Development of a rapid, cost effective, and point of care (POC) test used to quantify %HbS before, during and after transfusion therapy would ensure that current and accurate results are available to the healthcare provider when they are most valuable for decision-making. Several platforms have been developed to offer improved evaluations of SCD treatment[16-18](#_ENREF_16). We previously reported the development of a lateral flow immunoassay (LFIA) test[15](#_ENREF_15) to quantify and monitor HbS levels for patients going transfusion therapy as a SCD treatment. In this paper, we develop the technology of the POC quantitative HbS test and compare the LFIA test results with results from hemoglobin electrophoresis for 38 whole blood samples from SCD patients.

**PROTOCOL:**

This protocol follows institutional review board guidelines for ethical human research.

1. **Preparation for Testing**
   1. Prepare test kit materials: collect Cartridge, Capillary Sampler, and Pretreatment Buffer Module, as well as materials needed for blood draw (K2-EDTA vacutainer, alcohol swab, syringe, tourniquet, and bandage).
   2. Turn on the reader via the power button located on the lower left side of the unit. Wait approximately 5 min for the software to boot and device to perform self-check.
   3. When prompted, enter or barcode scan a User ID specific to the individual operator.
   4. Press TEST on the reader touchscreen to be ready to run test.
2. **Blood sample collection**
   1. Collect venipuncture whole blood samples from a patient who has the Hb genotype of HbSS and is to undergo transfusion treatment. Follow clinical protocols, but briefly:
      1. Select a large, firm vein, preferably in the antecubital fossa.
      2. To make the vein more prominent, apply a tourniquet and ask the patient to form a fist.
      3. Use 70% alcohol swabs to cover the whole area and ensure that the skin area is in contact with the disinfectant.
      4. Enter the vein at a 30° angle or less, and continue to introduce the 18 gauge (or size recommended by the institution’s operating procedures for blood draw) needle along the vein at the easiest angle of entry.
      5. Once sufficient whole blood (~2 mL) has been collected in a K2-EDTA anticoagulant vacutainer tube, release the tourniquet.
      6. Withdraw the needle gently and apply gentle pressure to the site with a clean gauze or dry cotton-wool ball.
      7. Immediately after withdrawal, invert the vacutainer tube 3 times.
      8. If the test will not be run within 4 hours, store the vacutainer tube in 2-8 °C. Otherwise store the vacutainer tube at room temperature.
3. **Testing procedure**
   1. Collect a small volume of whole blood sample (5 µL) in the Capillary Sampler provided in the test kit.
   2. Add the sample to the module containing proprietary PreTreatment Buffer immediately before testing.
   3. Invert the module three times to cause cell lysis and to release hemoglobin.
   4. Add 5 drops (100 µL) of the buffer-treated sample immediately onto the application site of the cartridge.
   5. Insert the Test Cartridge into the reader when prompted. Slide the Test Cartridge in until it ‘clicks’ into place. The reader will automatically detect the barcode on the Test Cartridge with test function and calibration curve for the specific lot inserted.
   6. Allow the test to run for 15 min with on-board timer in the reader for adequate detection and quantification of HbS. The %HbS will be shown on the screen. The output %HbS value is based on the inserted automated image analysis algorithm, which utilizes the colorimetric absorbance within specified areas of the test strip.
4. **Lot Verification Procedure**

Note: A barcode that labels each Test Cartridge includes information about test name, calibration curve algorithm, lot number, and expiration date. If the current date exceeds the expiration date, the reader provides the user a warning that results may not be valid.

* 1. When new lot of reader or cartridges is received, perform Lot Verification to ensure proper performance of this or any IVD reagent. Run External Controls 1, 2, and 3 as in Section 3.
  2. Record the output results. If the output results are in the range of what is indicated for the External Controls, the Test and Reader is ready for use.
     1. If External Controls do not report in the appropriate range, test the External Controls again. If the results are still not in the appropriate range, contact technical support.

1. **Clinical Application**
   1. Acquire general health information regarding the patient (i.e. age, gender, body weight, previous post-transfusion %HbS, other) and test for % HbS.
   2. Apply transfusion (exchange transfusion or simple transfusion).[19](#_ENREF_19)
   3. Test %HbS before each blood pack is transfused into the patient. Keep monitoring and transfusing blood packs until the target %HbS in patient is achieved.
   4. Once finished, record the post-transfusion %HbS to help physicians determine the time for next appointment.

**REPRESENTATIVE RESULTS:**

To enable the use of current and accurate results in the treatment of SCD patients, we have developed a POC test to quantify %HbS before, during, and after transfusion therapy. Our device applies the updated technology[20](#_ENREF_20) of newly developed rabbit anti-human HbS monocloncal antibodies and a small quantitative reader to a highly-accessible LFIA format seen commonly in pregnancy tests and flu tests.

The HbS-LFIA cartridges have been packaged in a heat-sealed aluminum pouch with desiccant to protect the device from sunlight and humidity. The HbS-LFIA cartridge and other materials in the HbS quantitative test set (module with pretreatment buffer and 5 µL capillary sample) are one-time use. The test system also consists of a point-of-care reader.

A capillary sampler is used to transfer sample to a module prefilled with pretreatment buffer (Figure 1). Mixing the sample and pretreatment buffer lyses the blood cells which releases the hemoglobin. Blood samples may be drawn from patients via fingerstick or venipuncture depending on what the healthcare provider determines to be less invasive. Once mixed with pretreatment buffer, 5 drops (100 µL) of treated sample is added to the cartridge’s application site. After a 15-min development period, the test is read using the small, quantitative reader. Analytical studies have previously been done to develop a concentration calibration curve between %HbS and colorimetric absorbance, and determine the limit of detection, the interference factors, and the effect of hemoglobin concentration for the HbS-LFIA test[15](#_ENREF_15).

**Procedure of test**

During the 15-min development, the treated blood sample moves through the series of laminated porous layers of the test strip that have been printed or sprayed with reagent and dried (Figure 2a). As the test sample diffuses through the absorbent test strip, the labeled mouse anti-hemoglobin (Hb) antibody - blue bead nanoparticle conjugate binds to the Hb in the specimen forming an antibody-antigen complex (Figure 2b). The specimen then migrates across a membrane toward the test line region containing HbS antibody to selectively detect the levels of HbS. The specific complex with HbS is captured at the test line and produces a blue band. The blue color intensity varies due to different amounts of HbS in each specimen. Excess conjugate will flow past the test line region and be captured at the control line region containing goat anti-mouse antibodies directed against any complex with mouse anti-Hb (Figure 2c). To serve as a procedural control, a blue band will always appear at the control line region if the proper volume of samples has been added and membrane wicking has occurred.

**Software Setup**

Each Reader is connected to a local PC through an Ethernet network for initialization. A software package is uploaded to each reader specifying parameters, such as the number of test lines to be quantified, tolerances for test line position, tolerances for control line position, and time before reading.

A calibration curve is established by using blood standards (Hemoglobin A0 and Hemoglobin S, Ferrous Stabilized human lyophilized powder) to test each new lot of HbS-LFIA tests. A calibration curve is established for each new lot of tests produced. The calibration curve between %HbS and colorimetric absorbance (test line peak value / control line peak value) is inserted into the automated image analysis algorithm and “%HbS” is set as the output format.

Lot-specific assay and calibration curve information are contained within the barcode that labels each Test Cartridge. The Reader will identify the test and insert calibration information for test type and test lot.

**Clinical study - Method Comparison**

A total of 38 whole blood samples (5 HbSC, 33 HbSS) ranging from 0-92.5% HbS were tested by both HbS-LFIA and central laboratory Hb electrophoresis methods (see Table 1). Figure 3 describes the high linear agreement between the data from the HbS-LFIA test and the data collected clinically from Hb electrophoresis. The whole blood samples ultimately yielded a linear fit within close proximity to the calibration curve (slope = 0.9949) and a high correlation (R2 = 0.9751) between the HbS-LFIA and the Hb electrophoresis data sets. The strong agreement in the data sets is seen in the Bland-Altman plot (Figure 3b), with 89.5% of samples falling within ±5% HbS difference. Between the clinically collected and HbS-LFIA data, the limits of agreement are ± 5.2% (± 2 SD). The strong agreement the HbS-LFIA and Hb electrophoresis results shows that the HbS-LFIA test has the ability to accurately measure % HbS within (0% to 93.4%) clinical approximation.

**Figure 1. Assay Workflow.**

(a) A small amount (5 µL) of sample is added to the module containing pretreatment buffer. (b) The module is inverted three times to mix the sample and pretreatment buffer. (c) The module cap is removed and 5 drops of diluted sample are added to the application site of the cartridge. (d) The reader allows the test to run for 15 min before reading the test and quantifying % HbS.[15](#_ENREF_15)

**Figure 2. Diagram of the HbS-LFIA lateral flow test strip.**

The scale unit is 5 mm. (a) Treated sample is added to the sample pad, which overlaps the conjugate pad. (b) As the sample flows through the conjugate pad HbS binds mouse anti-Hb antibodies conjugated to blue nanoparticles. (c) As the sample continues across the nitrocellulose membrane, HbS bound to conjugated blue nanoparticles will bind anti-HbS antibodies at the test lines. Unbound detector particles will pass these antibodies and bind the control line as the treated sample draws towards the absorbent pad.[15](#_ENREF_15)

**Figure 3. Comparison of the HbS-LFIA and Hb electrophoresis results for HbS measurements of 38 whole blood samples.**

(a) This linear plot illustrates the linear agreement and correlation between the % HbS determined by using the quantitative HbS-LFIA and Hb electrophoresis. The red dashed line represents the high correlation while the solid line displays a regression for the data trend. (b) The Bland-Altman plot illustrates the agreement between the results of the two quantitative methodologies. The red dashed line indicates the limit of agreement while the solid line indicates the difference, or mean bias, between the results of the two methods. The standard deviation (SD)=2.6% and the standard error of sample mean from the population mean (SEM)=3.3%.

**Figure 4. Comparison of the transfusion and decision making processes with and without the HbS-LFIA.**

(a) Without the use of the HbS-LFIA, SCD patients will visit the clinic and their blood will be drawn. Then, if the patient had blood drawn at their previous visit, the patient will receive transfusion guided by the HPLC results of the blood drawn during the previous visit. (b) With the use of the HbS-LFIA, upon arrival at the clinic, %HbS in SCD patients will be quantified and used in the immediate decision of whether or not to proceed with transfusion. If the physician decides to move forward with transfusion, %HbS will be routinely checked throughout the process until the appropriate %HbS is reached and transfusion can be terminated.

**Table 1. Pre-treatment Buffer specific composition.**

**Table 2. HbS comparison data.**

Hemoglobin electrophoresis results from Duke University for percent A, S and other hemoglobin compared to percent hemoglobin S results as determined by the HbS-LFIA test.

**DISCUSSION:**

The major goal of chronic RBC transfusion for SCD patients is to maintain a low %HbS (<30%) in order to reduce the rate of stroke and other severe complications.[7](#_ENREF_7),[21](#_ENREF_21) Generally, the chronic exchange transfusion of 2-4 RBC units every 3-5 weeks is sufficient to keep the %HbS less than 30% and the [Hb] at 9-10 g/dL, thereby reducing the severe complications of SCD.[7](#_ENREF_7),[21](#_ENREF_21) The transfusion frequency and volume varies for each individual, based on the patient’s historical pre-transfusion %HbS and the change in %HbS observed after previous transfusion. However, healthcare providers may have very little information, such as the patient’s initial %HbS level and their response to the administered RBCs, when treating SCD patients who need acute transfusion due to severe anemia, acute splenic sequestration, acute chest syndrome, or other acute organ damage.

Current methods of determining %HbS require highly-specialized HPLC or capillary electrophoresis equipment in a central laboratory with highly-trained technicians. These methods must navigate the laboratory testing infrastructure within large hospitals, often resulting in turn-around times of days to weeks. Thus, healthcare professionals utilize data from previous visits, weeks to months prior to the current visit, to estimate the number of blood packs that a patient may require.

Our goal is to eliminate the uncertainty of %HbS during transfusion (Figure 4a) for patients with SCD by implementing the HbS quantitative test in order to monitor %HbS to evaluate the need and appropriate volume for transfusion therapy at the bedside (Figure 4b). Between transfusions, calculating the increase in %HbS between two chronic transfusions (difference in %HbS from before current transfusion and after previous transfusion) gives the healthcare provider insight into the rate of %HbS increase during the inter-transfusion period. This information can guide both the volume and frequency of transfusion, which can in turn improve the quality of care given to a patient by reducing unnecessary transfusion and helping to prevent transfusion-related complications and stroke recurrence. It is critical to perform the lot verification procedures beforehand.

The use of the HbS-LFIA quantitative test for the assessment of the %HbS during blood transfusion represents a solution for determining the appropriate volume for transfusions in SCD patients. This technology aims to ensure SCD patients receive a sufficient amount of normal RBCs, reduce the risk of iron overload, and reduce burden on financial resources and time required to perform transfusion. In the future, we hope to utilize this technology to add additional test cartridges to quantify other important hemoglobin variants, such as HbF for patients undergoing hydroxyurea therapy.

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**DISCLOSURES:**

The authors declare the following competing financial interest(s): J.S.K., T.D.O., and X.Y. are employed by BioMedomics, Inc., which owns the patent for the testing device and therefore have a financial interest in the manuscript and test development.

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