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

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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. 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). Our point-of-care (POC) lateral flow immunoassay quantifies the %HbS in only 15 minutes 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 well, 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. All of the calibration for quantifying %HbS is built into the device, allowing for an automated quantification of %HbS with minimal maintenance by the user. 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.

Author Comments:	Dear Editor, Attached is a response to the most recent editorial comments. We thank you for your review. "1. Please tone down the language to not appear as product literature: in only 15 minutes using a small patient blood sample All of the calibration for quantifying %HbS is built into the device, allowing for an automated quantification of %HbS with minimal maintenance by the user. 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. The HbS-LFIA cartridges are packaged in a sealed aluminum pouch with a desiccant packet to ensure that the device is protected from sunlight and stored in low humidity. The HbS-LFIA cartridge and all other materials in the HbS quantitative test set (module with pretreatment buffer and 5 µL capillary sample) are one-time use materials. BioMedomics' HbS quantitative test set also includes a small desktop reader. The use of the HbS-LFIA quantitative test for the direct and unambiguous assessment of the percentage of HbS during blood transfusion represents an innovative solution for determining the need and appropriate volume for transfusion on a case-by-case (personalized medicine) basis. This ensures that patients receive a sufficient amount of normal RBCs, reduces the risk of iron overload, and prevents waste of RBC units, money and time required to perform transfusion." This language has been modified as requested. "2. Some additional details are needed: 1.1: Prepare test kit materials how? Thaw? 1.2: Wait how long for the boot and self-check? 2.1.4: What size needle? 3.1: What sampler? 3.2: What is this buffer used? Please provide the composition or provide the commercial information in the Materials Table." Modified and clarified as requested. "4: What is actually done in this section? 4.1: Please revise for grammar. 4.3: How are the external controls run? 5.2: How are the transfusions done? If this is to be filmed, we need explicit details. If this is not to be filmed, a citation would suffice. 5.3: ends or expires? 6: How are these calculations and plots done? What are these plots? Please provide citations for each and do not highlight these steps for filming." Modified as requested. Transfusions protocols vary from healthcare system to healthcare system. We would recommend demonstrating testing, showing very briefly the prep for transfusions, but would be wary to guide on how therapeutic procedures should be done. Removed protocols for calculations and plots. "3. There are many items used in the Protocol that are missing from the Materials Table: vacutainers, needles, etc. Please provide all materials and equipment used in the Materials Table." Blood draws are considered a separate clinical procedure by the FDA and CMS. We would be wary to guide on how a blood draw should specifically be done as these procedures may vary from different manufacturers (that are responsible for FDA approval) and also may slightly vary in healthcare systems. "Instead Tables 1 and 2 are uploaded twice. Please upload each Table individually." Updated as requested. "4. Please revise the Discussion to be more neutral and discuss the following in depth

	with citations (3-6 paragraphs): a) Critical steps within the protocol b) Any modifications and troubleshooting of the technique c) Any limitations of the technique d) The significance with respect to existing methods e) Any future applications of the technique" Updated as requested with additional comments for recommended topics.
Additional Information:	
Question	**Response**
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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, R^2 = 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] Approximately two out of every 1000 births worldwide and more than 10 of every 1000 births in Africa are affected by SCD.[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] 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], all of which contribute to vaso-occlusion, as well as to complications such as strokes and acute chest syndrome.[3]

The severity of SCD varies greatly amongst individuals and correlates with a number of both hematologic and non-hematologic factors.[5,6] 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] 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]. 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.[7,8]

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], high performance liquid chromatography[12], or isoelectric focusing[13,14]. 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].

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]. We previously reported the development of a lateral flow immunoassay (LFIA) test[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

1.1. 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).

1.2. 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.

1.3. When prompted, enter or barcode scan a User ID specific to the individual operator.

1.4. Press TEST on the reader touchscreen to be ready to run test.

2. Blood sample collection

2.1. 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:

2.1.1. Select a large, firm vein, preferably in the antecubital fossa.

2.1.2. To make the vein more prominent, apply a tourniquet and ask the patient to form a fist.

2.1.3. Use 70% alcohol swabs to cover the whole area and ensure that the skin area is in contact with the disinfectant.

2.1.4. 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.

2.1.5. Once sufficient whole blood (~2 mL) has been collected in a K2-EDTA anticoagulant vacutainer tube, release the tourniquet.

2.1.6. Withdraw the needle gently and apply gentle pressure to the site with a clean gauze or dry cotton-wool ball.

2.1.7. Immediately after withdrawal, invert the vacutainer tube 3 times.

2.1.8. 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

3.1. Collect a small volume of whole blood sample (5 µL) in the Capillary Sampler provided in the test kit.

3.2. Add the sample to the module containing proprietary PreTreatment Buffer immediately before testing.

3.3. Invert the module three times to cause cell lysis and to release hemoglobin.

3.4. Add 5 drops (100 µL) of the buffer-treated sample immediately onto the application site of the cartridge.

3.5. 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.

3.6. 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.

4.1. 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.

4.2. 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.

4.2.1. 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.

5. Clinical Application

5.1. Acquire general health information regarding the patient (i.e. age, gender, body weight, previous post-transfusion %HbS, other) and test for % HbS.

5.2. Apply transfusion (exchange transfusion or simple transfusion).[19]

5.3. 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.

5.4. 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] 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].

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 (R^2 = 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]

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]

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.[7,21] 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.[7,21] 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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Figure1

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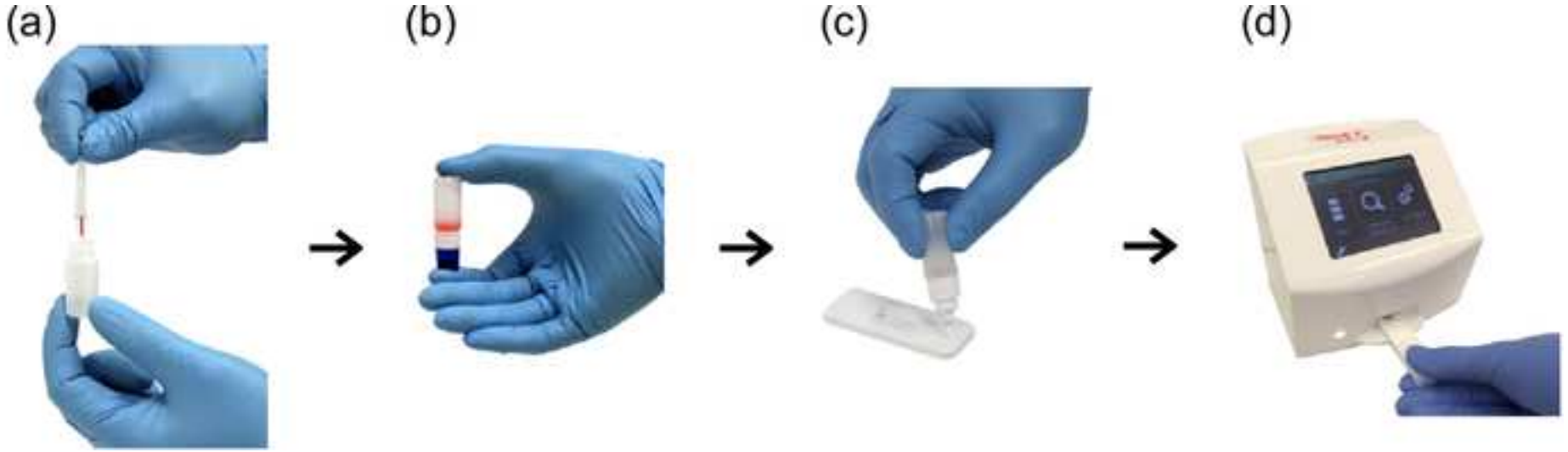

Figure2

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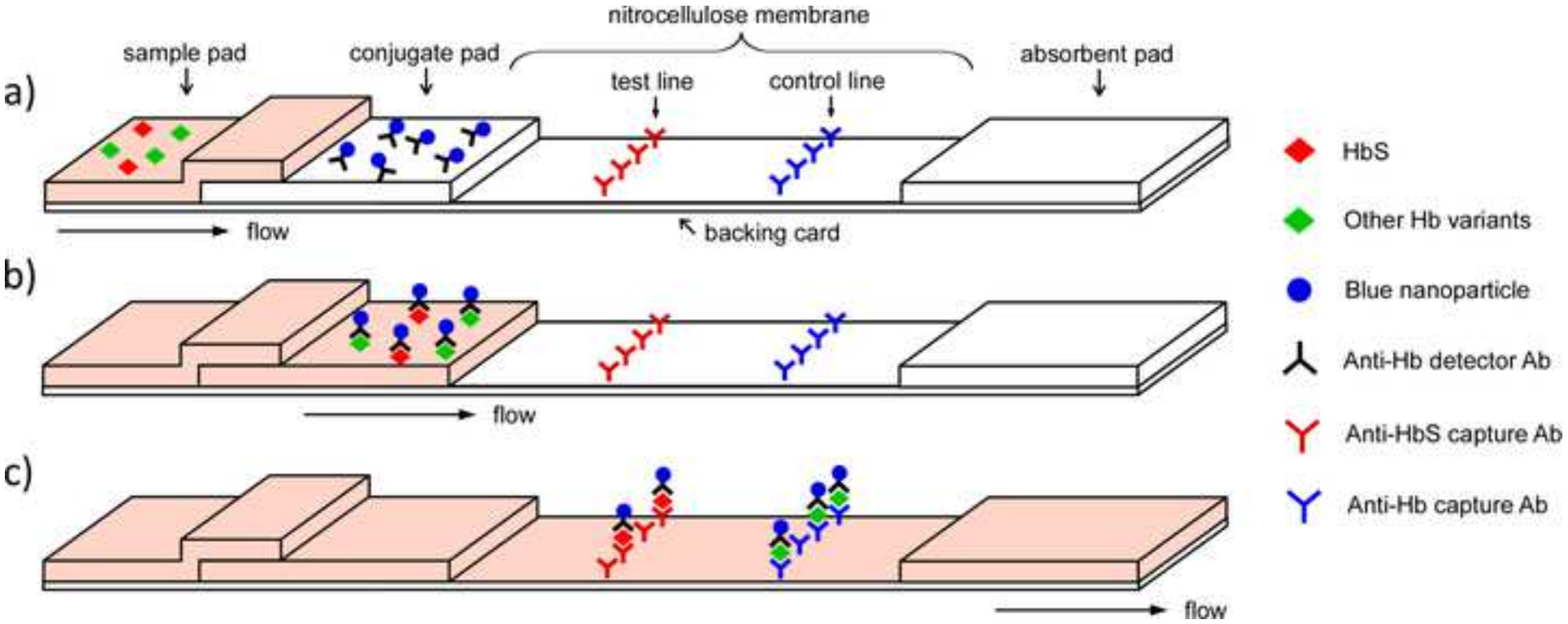

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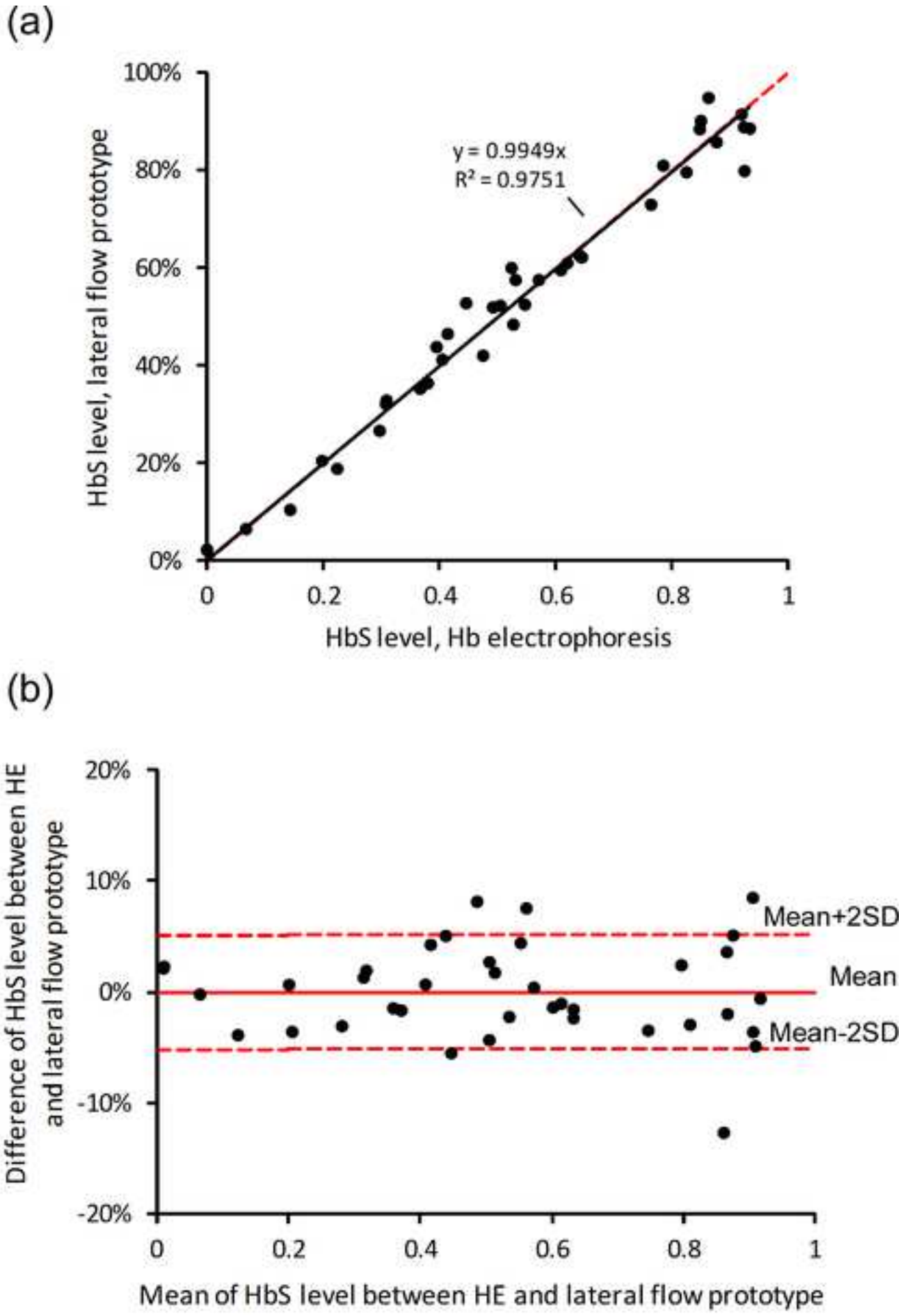

Figure4

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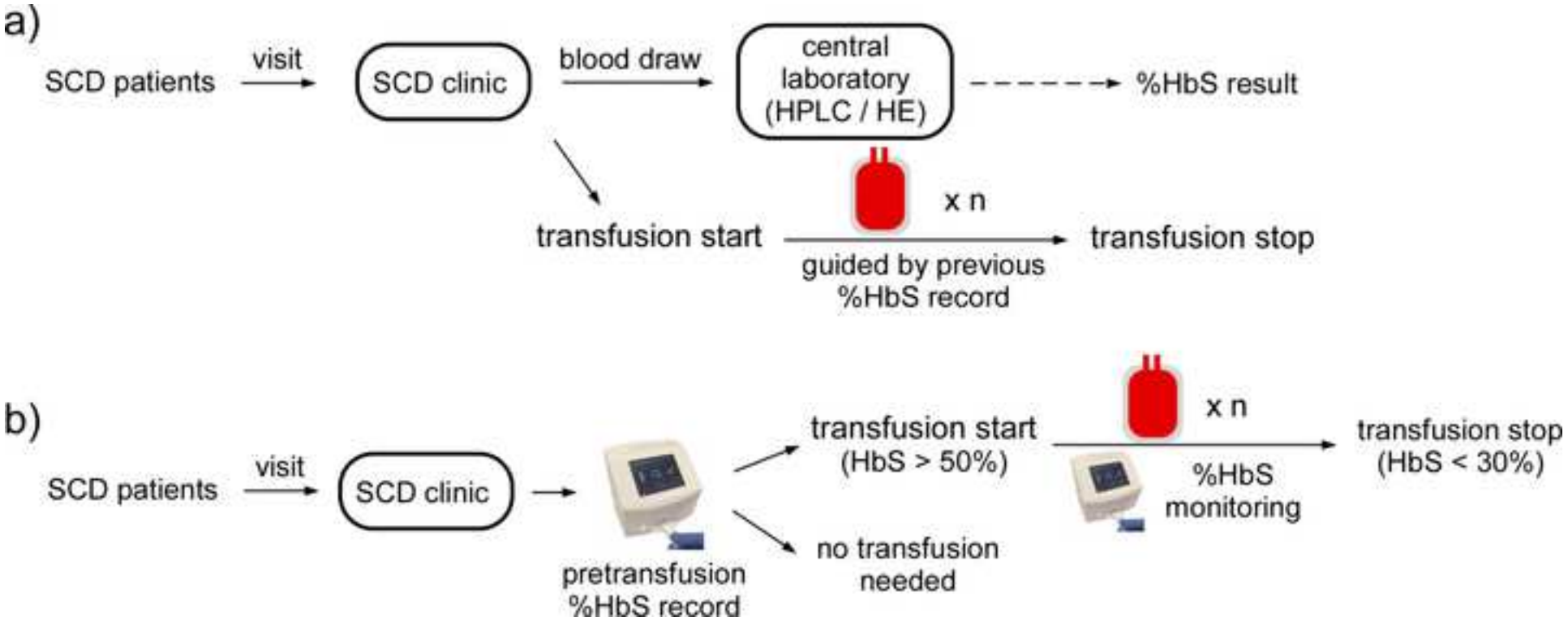

Table 1

Composition (specific chemical identity)		
Ingredients	Cas Number(s)	Proportion (%)
Polyoxyethylene (4) lauryl ether	9002-92-0	<2
Ethylenediamine	26316-40-5	<2
Sodium borate decahydrate	1303-96-4	<1
Sodium azide	26628-22-8	<0.05
Hazards identification		
OSHA PEL	No ingredients are hazardous according to OSHA	

Table 2

Click here to download Table HbElect Data Table.xlsx

Sample #	Hemoglobin Electrophoresis			%HbS, LFIA	%HbS, Method Bias
	% HbA	% HbS	% Other		
1	65.8	30.8	3.4	32.1	1.3
2	6.8	84.8	8.4	88.4	3.6
3	55.7	40.5	3.8	41.2	0.7
4	0	92	8	91.4	-0.6
5	0	92.4	7.6	88.8	-3.6
6	30.3	60.9	8.8	59.5	-1.4
7	47.8	49.2	3	51.9	2.7
8	0	92.5	7.5	79.8	-12.7
9	57.2	39.5	3.3	43.7	4.2
10	0	93.4	6.6	88.5	-4.9
11	12.9	44.6	42.5	52.7	8.1
12	0	78.5	21.5	80.9	2.4
13	42.8	52.4	4.8	59.9	7.5
14	37.7	53.1	9.2	57.5	4.4
15	15.1	76.4	8.5	72.9	-3.5
16	90.2	6.7	3.1	6.5	-0.2
17	65.6	29.7	3.7	26.6	-3.1
18	19.8	41.4	38.8	46.4	5.0
19	31.1	64.1	4.8	62.5	-1.6
20	77	19.8	3.2	20.4	0.6
21	37.6	57.1	5.3	57.5	0.4
22	0	85	15	90.1	5.1
23	0	87.7	12.3	85.7	-2.0
24	0	86.3	13.7	94.8	8.5
25	65.9	30.9	3.2	32.8	1.9
26	73.8	22.4	3.8	18.8	-3.6
27	60.4	36.7	2.9	35.2	-1.5
28	0	50.5	49.5	52.2	1.7
29	47.8	47.5	4.7	42.0	-5.5
30	83	14.3	2.7	10.4	-3.9
31	71.7	0	28.3	2.1	2.1
32	63	0	37	2.3	2.3
33	15	64.5	20.5	62.1	-2.4
34	38.2	52.7	9.1	48.4	-4.3
35	0	82.5	17.5	79.5	-3.0
36	56.6	38	5.4	36.3	-1.7
37	21	62	17	60.9	-1.1
38	40.6	54.7	4.7	52.4	-2.3

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment
JoVE_Materials (121416).xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
HbS test kit	BioMedomics	HLX020	Includes capillary samplers and pretreatment buffer modul
BioMedomics Quantitative Reader	BioMedomics	XJF-M	
PreTreatment Buffer	BioMedomics	PT001	Contained within Pretreatment Module
K2-EDTA anticoagulant vacutainer tube	BD	367835	Please use as per Manufacturer instructions or your institu
Sterile Alcohol Prep Pads	Fisher Scientific	22-363-750	Please use as per Manufacturer instructions or your institu

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Title of Article: A PRECISION MEDICINE TOOL FOR MEASUREMENT AND MONITORING OF HEMOGLOBIN S IN SICKLE CELL DISEASE PATIENTS RECEIVING TRANSFUSIONS

Author(s): XIAOXI YANG, TAYLOR D. OSBORNE, MARTHA DELAHUNTY, MARILYN J. TELEN, JASON S. KIM

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CORRESPONDING AUTHOR:

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Department: PRODUCT DEVELOPMENT

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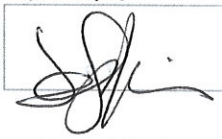
Article Title: A PRECISION MEDICINE TOOL FOR MEASUREMENT AND MONITORING OF HEMOGLOBIN S IN SICKLE CELL DISEASE PATIENTS RECEIVING TRANSFUSION

Signature: 

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Response to the review comments on the manuscript #55607_RO_102016 "*A Precision Medicine Tool for Measurement and Monitoring of Hemoglobin S in Sickle Cell Disease Patients Receiving Transfusion Therapy*"

We appreciate the insightful comments from the reviewers. We have revised the manuscript accordingly and would like to take this opportunity to clarify some details and elaborate the significance of our work.

In the following parts, we will address each individual question raised by the reviewers.

Editorial comments:

Line 49: Is Red blood cell transfusion the only therapy for SCD? If not then in line 49 please mention "one of the…most commonly used…".

Response: Editor is correct that the red blood cell transfusion is not the only therapy for SCD. Other therapies include hydroxyurea therapy as well as bone marrow transplant. However, red blood cell transfusion is the most commonly used therapy to treat or prevent these devastating complications. We included other therapies in the Long Abstract.

Line 57: Please remove "well,".

Response: We removed the term as the editor requested.

Lines 112-159: Please adjust the numbering of your protocol section to follow JoVE instructions for authors, 1. should be followed by 1.1) and the n 1.1.1) if necessary and all steps should be lined up at the left margin with no indentations.

Response: We formatted the section as the editor requested.

Lines 112-145: Please add a one line space between each step and sub-steps of your protocol section.

Response: We formatted the section as the editor requested.

Lines 112-145: Please re-write steps of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Please try to avoid usage of phrases such as “should be”, “could be”, “would be” and write in the active/imperative style. For example, “Connect a small quantitative reader…”,etc.

Response: We changed the terms as the editor requested.

Line 118: How are all these parameters set?

Response: The number of test lines is set to one because the HbS-LFIA strip has one test line and one control line. By running a sample strip with the correct line position, the position of the test line and control line is recorded in the local PC associated with the quantitative reader as the ideal line. The search region is defined as the region that just encloses the ideal line and also marks the boundary of the search area.

Line 119: How is the calibration curve inserted? How is this curve established in the first place? Are these to be done each time the device is turned on?

Response: The calibration curve is inserted to the automated image analysis algorithm and transferred to the quantitative reader via a USB key. The calibration curve is established by using blood standards (Hemoglobin A0 and Hemoglobin S, Ferrous Stabilized human lyophilized powder, Sigma) to test each new lot of HbS-LFIA tests prodouced. Establishing a calibration curve only needs to be done once per new lot of tests produced.

Lines 122-124: Please provide details on how these are executed. Please be aware that steps that involve coding cannot be filming.

Response: We added more details as the editor requested.

Lines 112-145: For steps that involve software, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. This is applicable throughout the protocol that uses any form of Graphic user interface or a software program.

Response: We added more details as the editor requested.

Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.

Response: We added requested details.

Line 126: In step 2.a please provide all the details of the venipuncture, these should include sterilization of the local area, the tools used and approximately how much volume of Blood is withdrawn.

Response: We added more details as the editor requested.

Line 129: In step 2, b. following collection how are the tubes stored? Are the tubes shaken?

Response: We added more details as the editor requested.

Line 133: In step 3 b, what are the contents of the Pre-treatment buffer? Is this prepared in house? Please make sure to list out all the reagents and materials in the spreadsheet.

Response: The Pre-treatment buffer is prepared in house. We included a Table in excel file and uploaded it to the Editorial Manager site as the editor requested.

In section 3 are there any functions that need to be pressed or selected to execute the test. Please ensure to provide more details.

Response: We added more details as the editor requested.

After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10 page limit for the protocol text, but there is a 2.75 pages limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages (or less) of text to identify which portions of the protocol are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Response: After making all of the recommended changes, the protocol section is less than 2.75 pages and can all be included in the video.

Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

Response: We removed the embedded figures and uploaded them to the Editorial Manager site in the form of a Word document.

Please remove the embedded Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends

Response: We removed the embedded table and uploaded it to the Editorial Manager site in the form of an Excel file.

Please place the “Representative results section” section before the “Figure legends” section after the “Protocol” section.

Response: We relocated this section as requested by the editor.

Please expand your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.

Response: In effort to highlight the importance of this new technique, we have added a “Clinical Application” addition to our Protocol. We hope that this focuses the importance of this technique in quantifying %HbS to aid in guiding transfusion therapy for SCD patients.

Please place the “Figure Legends” section before the “Discussion” section after the “Representative results section”.

Response: We moved this section as requested by the editor.

Each figure or data table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description. All figures showing data must include measurement definitions and error bars (if applicable). Please include the figure legends as part of the manuscript text (not part of the figure file) directly below the representative results text.

Response: We included and relocated all figure legends as requested by the editor.

In the figures, please specify a scale bar and define the scale units in the figure legends.

Response: There is only one figure qualified to add a scale bar, Figure 2. We added a scale bar in Figure 2 and defined the scale unit in the figure legend.

Please define all error bars (SD, SEM) in the legends of their respective figures.

Response: We defined the error bars of the figure in the legend of figure 3 as requested by the editor.

Please provide the name of the statistical test carried out in the legends.

Response: We added the name of the statistical test in the legend of Figure 3 as the editor requested and added a section "statistical analysis" in Protocol.

Please make sure that the “Discussion” is written under the following sections.

a. Critical steps within the protocol.
b. Modifications and troubleshooting.
c. Limitations of the technique.
d. Significance of the technique with respect to existing/alternative methods.
e. Future applications or directions after mastering this technique.

Response: We checked our discussion section as requested by the editor.

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Response: We included the DOIs when available as the editor requested.

Reviewer #1:

The investigators in this report describe a point-of-care device for, measuring the concentration of HbS in patients with sickle cell disease. This device could potentially revolutionize the approach to patient care, particularly in settings where these patients require frequent blood transfusions to control disease severity.

1. The device has been tested utilizing only a limited number of blood samples. It is not clear what the test performance will be with more samples with varied hemoglobin concentration, i.e severely anemic and not so anemic patients with sickle cell disease regardless of the HbS concentration. The absolute hematocrit data would have been informative on the samples tested in this report.

Response: We agree that this data provides a lot of information pertaining to the performance of the test. We included data on the effect of hemoglobin concentration on test performance in our previous paper. Please refer to the "Representative Results" section where we cited our previous paper's information on the effect of hemoglobin concentration on the test.

2. The description of the use of this device as contained in the current report, refers to spot checks for Hb concentration, which presumably can be done before and after transfusion. While this provides meaningful information, in order to apply the use of the device to better guide the volume of blood transfused, it will be helpful to have data on the use of the device for continuous monitoring of HbS concentration during transfusion. Without such data, this device cannot be marketed as one that meets this need.

Response: We believe the reviewer means to write "HbS concentration" in the comment when the reviewer accidently writes "Hb concentration". The reviewer is correct in that monitoring the extent of %HbS decline for patients with SCD during transfusion (and especially during exchange transfusions) would help evaluate the appropriate volume needed during transfusion and when to stop transfusion according to current standard of care. We have already included the statement in the Discussion.

3. There is a strong argument for short turnaround time and cost effectiveness, but there is no discussion on how the cost of this device will compare with existing platforms in developing countries where, this application will be most relevant

Response: We agree that this technology would be extremely useful in the developing world where sickle cell is so prevalent. However, transfusion is not currently widely available as a treatment for sickle cell patients in the developing world. We hope that eventually the application of our technology in the developing world will assist in transfusion treatment becoming more widely available and accessible.

Recommendation: Publish either as is or subject to minor revisions as indicated.

Comments: This is a clearly presented and potentially very useful technique for improving transfusion efficacy and safely in sickle cell disease patients. Some small points in the narrative deserve clarification.

Reviewer #2:

Manuscript Summary:

This paper is well written and of strong interest.

The advent of a new device for HbS detection would be very welcome for patients and clinicians.

Needs to add emphasis on saving cost (testing) and reducing blood transfusion (alloimmunization and costs) and improving compliance (self monitoring)

Response: We added to the discussion to emphasize saving cost and reducing blood transfusion cost. As the purpose of this test is for monitoring patients during treatment, there will be no need

for patients to be able to self-monitor. The device is for healthcare professionals to use while administering transfusion treatment to patients.

Major Concerns:

- SHORT ABSTRACT & LONG ABSTRACT: needs to stress the advantage of POC versus standard HPLC.

For example will POC become a portable device?, would be possible in the future for patients to perform self monitoring such as for coagucheck (patients on warfarin) or for diabetic patients??

Response: The reviewer correctly notes strong interest in home-use or self-monitoring for SCD patients. While our group is also interested in this application, we have encountered many regulatory hurdles and questions regarding self-testing that requires further consideration. We believe that near-patient testing to guide on-site transfusion therapy will increase familiarity with this technology to potentially allow for self-monitoring applications.

- INTRODUCTION: please add reference on pathophysiology of SCD row 77 (e.g. J Extracell Vesicles. 2015 Nov 23;4:28414. Circulating microparticles, protein C, free protein S and endothelial vascular markers in children with sickle cell anemia; Protein C and free protein S in children with sickle cell anemia. Ann Hematol. 2012 Oct;91(10):1669-71; The role of blood rheology in sickle cell disease. Connes P, Alexy T, Detterich J, Romana M, Hardy-Dessources MD, Ballas SK. Blood Rev. 2016 Mar;30(2):111-8)

Response: we included the reference (The role of blood rheology in sickle cell disease. Connes P, Alexy T, Detterich J, Romana M, Hardy-Dessources MD, Ballas SK. Blood Rev. 2016 Mar;30(2):111-8) on pathophysiology of SCD as the editor requested.

88-90: add also that transfusion reduces HbS production in bone marrow

Response: We thank the Reviewer for the valuable input. However, to the best of our knowledge, there is no direct proof that transfusion reduce HbS production in bone marrow.

-DISCUSSION: please try to quantify economical advantages and disadvantages, costs of testing patients with POC versus HPLC.

Response: We added emphasis on cost saving and economic advantages to the discussion as requested by the reviewer.

Move the picture after discussion title into method/protocol

Response: We moved this section as requested by the reviewer.

Reviewer #3

Manuscript Summary:

Laudable goals and interesting, practical methodology.

Not clear how percentage HbS is calculated (is there comparison to the quantitation in the control band, so %S = S/(S+control)?

Response: The percentage HbS is calculated by the inserted calibration curve between %HbS and colorimetric absorbance (test line peak value / control line peak value). The calibration curve is established by using blood standards (Hemoglobin A0 and Hemoglobin S, Ferrous Stabilized human lyophilized powder, Sigma) after each lot of HbS-LFIA test. It only needs to be done once when new lot of tests is received. We added this information in the Protocol.

Demonstration is needed of serial values in individual patients undergoing transfusion compared to serial values by a standard method.

Response: In this manuscript, we did analytical study to compare the HbS-LFIA test results with the results from hemoglobin electrophoresis - Sebia Minicap Hemoglobin(E) kit - the standard method for 38 whole blood samples from SCD patients. We agree with the reviewer that the comparison of the two results for samples from individual patients undergoing transfusion in clinical study would be a stronger demonstration for the application we proposed. Since this manuscript aims to investigate the feasibility of the technology of the POC quantitative HbS-LFIA, we believe, however, that the clinical study is outside of the scope of this manuscript.

Major Concerns:

Is method to be used with serial total hemoglobin levels done in parallel?

Response: In current clinical transfusion setting, Hb concentration is needed to calculate the appropriate volume to transfuse. However, the HbS-LFIA test does not need the assistance of information of the total hemoglobin levels. However, we agree with the reviewer that knowing the total hemoglobin levels could be helpful in the monitoring process.

Minor Concerns:

In introduction, first paragraph, statement "When inherited, the Hb S gene causes mutation in the beta chain...." This is not accurate. Better: Inheriting the Hb S gene results in production of abnormal beta globin chains that precipitate when deoxygenated etc etc..."

Response: We changed the terms as the reviewer requested.

In last paragraph of introduction:..."To quantify and monitor Hb S levels for patients undergoing SCD treatments" This sentence really pertains to transfusion rather than other SCD treatments and this setting should be explicitly stated.

Response: We added to the statement for clarity.

"…to quantify and monitor HbS levels for patients undergoing transfusion therapy as a SCD treatment."

In addition to the changes introduced in response to the comments of the Editor and Reviewers (above) we made several minor corrections throughout the revised manuscript to improve its readability.