**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.

JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

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.