Supporting Information

Density-based Separation in Multiphase Systems Provides a Simple Method to Identify Sickle Cell Disease

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Materials and Methods

Chemicals. We purchased the following polymers: poly(ethylene glycol) (Sigma-Aldrich; MW = 20000 Da), Ficoll (Sigma-Aldrich; MW = 70000 Da and 400000 Da), dextran (Spectrum Chemical; 500000 Da), and poly(vinyl alcohol) (PVA) (Polysciences; MW = 3000 Da)—formed by hydrolyzing 75% of poly(vinyl acetate). Solutions of AMPSs contained the following chemicals: ethylenediaminetetra-acetic acid disodium salt (EDTA) (Sigma-Aldrich), potassium phosphate monobasic (EMD), sodium phosphate dibasic (Mallinkrodt AR), sodium chloride (EMD), $MgCl_2$ (USB), and Nycodenz (Axis-Shield PoC). We used a Hemacolor Stain Kit (Harelco) to stain slides of thin smears of blood. For the nystatin treatment, we purchased the following additional chemicals: nystatin (*Streptomyces noursei*, Calbiochem), choline chloride (Sigma-Aldrich), tris (hydroxymethyl) aminomethane hydrochloride (Tris HCL, Bethesda Research Laboratories), 3-(N-morpholino) propane-sulfonic acid (MOPS, EM Science), potassium chloride (EMD), sucrose (EMD), glucose (Sigma-Aldrich), albumin from bovine serum (Sigma-Aldrich), and sodium phosphate monobasic (Mallinkrodt Chemicals).

Blood Samples. Children's Hospital Boston (CHB) and the Sickle Cell Center of Southern Louisiana (SCCSL) (New Orleans) provided de-identified blood samples with known hemoglobin genotypes.

We tested our system on a variety of blood samples that were Hb AA, Hb AS, Hb SS, Hb SC, and one sample that was Hb $S\beta^+$. The Hb SS samples varied in their Hb F content and their proportion of dense sickled cells. We used de-identified blood from Research Blood Components in Boston for our model sickle blood systems.

At the SCCSL, blood samples were collected into 4 mL Vacutainer tubes (K_2EDTA, BD, Franklin Lakes, NJ) during routine blood draws from patients with informed consent, according

to a protocol approved by Tulane University Biomedical IRB. At CHB, blood samples were collected when clinically indicated and discarded samples were used according to a protocol approved by Children's Hospital Boston IRB.

Three normal controls were obtained from consented volunteers at Harvard University under a protocol approved by the Committee on the Use of Human Subjects at Harvard University.

There are many variations of genetic disorders and concurrent conditions that can affect the density of red blood cells (**Table S1**). For example, iron deficiency often leads to hypochromic, microcytic anemia (1); a patient with SCD and iron deficiency anemia may have a more complex distribution of densities of erythrocytes. Patients suffering from sickle-cell disease with alpha thalassemia trait and alpha thalassemia may have fewer dense cells (2). In a study by Bartolucci, et al., the amount of dense cells in those with one gene deletion (13% (S.D. 7%)) and those with gene triplication (17% (S.D. 5%)) was similar to the amount of dense cells in patients without alpha thalassemia 15% (S.D. 8%)(3). In the case of alpha thalassemia with two gene deletions, however, the percent of dense cells in SCD was reduced to 5% (S.D. 4%). Even in this case, the mean amount of dense cells present was above the limit of detection of SCD-AMPS (2.8%). In those with SCD and concurrent alpha thalassemia with a double deletion, however, the sensitivity of SCD-AMPS may be lower because the amount of dense cells present at one standard deviation below the mean is below the limit of detection. In this study, one of the Hb SS samples had alpha thalassemia and it was distinguishable both visually and digitally as SCD in our test.

Materials for Rapid Tests. We purchased the following materials to make our rapid tests: heparinized, polycarbonate microhematocrit tubes (Iris Sample Processing), white

Table S1. Geographic distribution and effect on density of sickle cell variants, concurrent conditions, and other conditions of relevance for density-based tests.

Population	Geographic Prevalence	Amount of Dense Cells[a]	Reference
Normal, Hb AA	Worldwide	Absent or below < 2%	(4)
SCD Variants			
Hb SS	sub-Saharan Africa, South Asia	Present in large amounts (> 10%)	(3, 5, 6)
Hb SC	West Africa	Potentially decreased compared with SS but still present	(6, 7)
Hb $S\beta^{0,+}$	Mediterranean, West Africa, South-East Asia	Potentially decreased compared with SS but still present	(6)
Hb SE, Hb SD, Hb SO	South-East Asia, South Asia	Present but not precisely quantified in previous studies	(6)
Concurrent Conditions			
iron deficiency anemia	World-wide	Potentially decreased cell density due to hypochromia	(1, 8)
Malaria	sub-Saharan Africa, South Asia, South-East Asia, Latin America	Unknown (parasite-infected cells have lower density, but effect on dense sickled cells is unknown). Anemia and hemolsyis may decrease the number of dense cells.	(9, 10)
alpha thalassemia	Mediterranean, West Africa, South-East Asia	In double alpha deletion, decreased compared with SS but still significant (~5%). Present in large amounts for other variants.	(3, 6)
Potential Sources of False Positives			
Hb CC	West Africa	Uniform increase in cell density, but densest cell fractions are fewer than those of SCD.	(7)
hereditary spherocytosis	Northern Europe	Present in large amounts, and reduced post-splenectomy.	(11)

[a] dense cells defined as cells with either a mass density > 1.120 g/cm^3 or a hemoglobin concentration > 41 g/dL depending on the study.

vinyl-based selant (Critoseal, Leica), silicone rubber tubing with an inner diameter of 1.02 mm and an outer diameter of 2.06 mm (Helix Mark, Helix Medical), and five-minute epoxy.

Preparation of AMPS. To prepare each AMPS, we added polymers, buffer salts, and other additives (i.e., Nycodenz and EDTA) in volumetric flasks and added deionized water to attain the final volume. Adjustments to pH and osmolality were made as described in the main text. A vortexer or magnetic stir bar mixed solutions thoroughly.

In our AMPSs, we included 5 mM EDTA and 1 mM $MgCl_2$ to help preserve the blood and prevent coagulation. The tubes were also heparinized. We have varied the amount of these additives but we have been unable to completely eliminate the clotting platelets.

Characterization. We measured density with a density meter (DM50, Anton Paar), osmolality with a vapor pressure osmometer (Vapro 5600, Wescor), and pH with a pH meter (Orion 2 Star, Thermo Scientific). Complete blood counts were done on a hematology analyzer (ADVIA 2120, Siemens).

Rapid Test Fabrication. We used a 3D printer (Fortus 250mc, Stratasys) to print a holder to punch reproducible holes in the sides of the microhematocrit tubes. The holder was designed with AutoCAD (AutoDesk). We load each holder with microhematocrit tubes and use standard metal pushpins (Staples) to punch holes in the sides of the tubes at the prescribed length. We also used fine tipped markers to mark a fixed point on the length of the tubes as fill lines to hold the prescribed volume of the AMPS. After removing the tubes from the holder, we blew out any loose plastic with an air gun. We cut small lengths of silicone tubing (3-5 mm) and slid them over the tubes to cover the holes in their sides. While an AMPS was being stirred by a magnetic stir plate, we used a micropipettor to fill the marked tubes up to the fill lines and then

sealed them with either white vinyl-based sealant or epoxy. The completed tests were then used on blood samples as described in the main text.

For larger productions, we estimated the costs necessary to cap and more permanently seal the tubes with glue as well as labor, equipment, and packaging costs (**Table S2**). Time estimates were based on current manufacturing procedures in the laboratory and materials costs were based on the volumes at which we currently purchased materials and chemicals. With these parameters, the cost per test is $0.50. Production in a market with lower labor costs and with bulk chemical prices should reduce this cost.

Nystatin Treatment for Model Sickle System. We created dehydrated erythrocytes using the nystatin loading procedure developed by Canessa (12). When nystatin is present, the membrane of erythrocytes becomes permeable and the volume of the cell can be set by adjusting the osmolality of the solution with additives like sucrose. Washing to remove the nystatin returns cells to a less permeable membrane while retaining the adjusted volume. Cells were washed five times in a choline wash solution of 150 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Tris HCl and MOPS with a pH adjusted to 7.4 at 4° C. We then exposed the cells to nystatin in a nystatin loading solution containing 10 mM NaCl, 130 mM KCl, and 200 mM sucrose for 20 minutes at 4° C. This solution was spun down and the supernatant removed. We incubated the cells in loading solution (without nystatin) for 10 minutes at 37° C followed by four washes with the loading solution at the same temperature. Finally, we washed the cells five times in the choline wash solution at 4° C. We suspended packed cells in homologous plasma at the same hematocrit as the original blood and made serial dilutions to attain a range of percentages of dense cells of blood from each donor.

Table S2. Itemized cost per test estimated for production.

Item	Unit Cost
Polycarbonate capillary tube	$ 0.1000
Critoseal	$ 0.0027
Critocaps	$ 0.0415
Silicone sleeve	$ 0.0079
Glue (Krazy Glue)	$ 0.0033
Polymer solutions	$ 0.0032
Foil-lined Pouch (12 devices/pack)	$ 0.0625
Total Consumable	**$ 0.2211**
Total Manufacturing Equipment & Personnel	**$ 0.2756**
Total Cost	**$ 0.4967**

Visual Evaluation of Rapid Tests. Samples from either Children's Hospital Boston or the Sickle Cell Center of Southern Louisiana were coded before being sent for evaluation by the rapid test at Harvard University. The samples were run on both SCD-AMPS-2 and SCD-AMPS-3 in duplicate and evaluated independently by two readers who had been trained on previous prototypes to read tests as positive when a full layer of red cells was present at the bottom of the microhematocrit tube. In instances where the duplicate samples gave different results, a third test was run with the same sample. In instances when the two readers disagreed on a result, a third trained reader evaluated the test independently. The two readers were in accordance on 97% of tests with SCD-AMPS-2 and 86% of tests with SCD-AMPS-3. Tests where the two original readers did not agree did not correlate to false positives or false negatives.

Digital Evaluation of Rapid Tests. To capture comparable digital images of all our rapid tests, we used a digital scanner in transmission mode (Epson Perfection V330 Photo) to record images of up to 12 tubes at a time placed in a plastic grid. We then used custom written Matlab code to process and analyze the images through several steps: i) scanned images were matched to a key image file using image registration and cropped to a standard size, ii) the matched images were cropped at twelve positions into separate image files for each tube, iii) images were converted into the Lab colorspace, iv) the region of interest that contained the bottom of the tube was selected, v) each pixel was evaluated for the intensity of the red color through a combination of intensity and distance in the Lab space from a training set of red, vi) the scores for all pixels were summed to give a single score for each tube, and vii) the calculated values for each tube were written to a file for further analysis and comparison.

The Lab colorspace is designed to approximate human vision so we chose to use this colorspace over other schemes, such as RGB and CMYK. We then defined a range of acceptable

red colors using a training set of sickle cell positive samples and using a weighting scheme to evaluate the distance in the colorspace from the learned red color. We used the "L" component, or lightness, to weight the density of the packed red cells so that darker packed red would count more strongly than a light red that was present when cells were not packed.

Statistical methods. Sensitivity is defined as (# true positives)/(# true positives + # false negatives). Specificity is defined as (# true negatives)/(# true negatives + # false positives). We chose to use Jeffreys confidence intervals because our values were near the upper bounds of 100% sensitivity or specificity.

Experimental Details

AMPS phases for separations. An AMPS with *n* phases has a total of *n+1* interfaces (AMPS/container, *n-1* AMPS phase/phase, and AMPS/air) at which to separate objects. For practical applications where blood is layered on top of an AMPS, and centrifugation is used to separate cells at the interfaces between the phases of the AMPS, the top (AMPS/serum) interface is diffuse and not useful for separations in this application; there are, therefore, *n* sharp interfaces that can concentrate cells.

Densities of erythrocytes determine the densities of the phases. A bottom phase, with a density of $\rho \geq 1.120$ g/cm^3, should permit dense, SCD cells ($\rho \geq 1.12$ g/cm^3) to sediment, while potentially creating a barrier to the dense cells of Hb CC blood ($\rho_{max} \sim 1.11$ g/cm^3) (7). Although we were unable to test blood with Hb CC due to the rarity of this blood type in the hemoglobinopathy clinics we worked with, we designed both systems with sufficiently dense bottom phases that future work with Hb CC could be done in areas (e.g., West Africa) with a higher prevalence of this genotype. The top phase must be less dense than low-density erythrocytes, such as reticulocytes ($\rho = 1.085$ g/cm^3), to ensure that all the erythrocytes pack at a

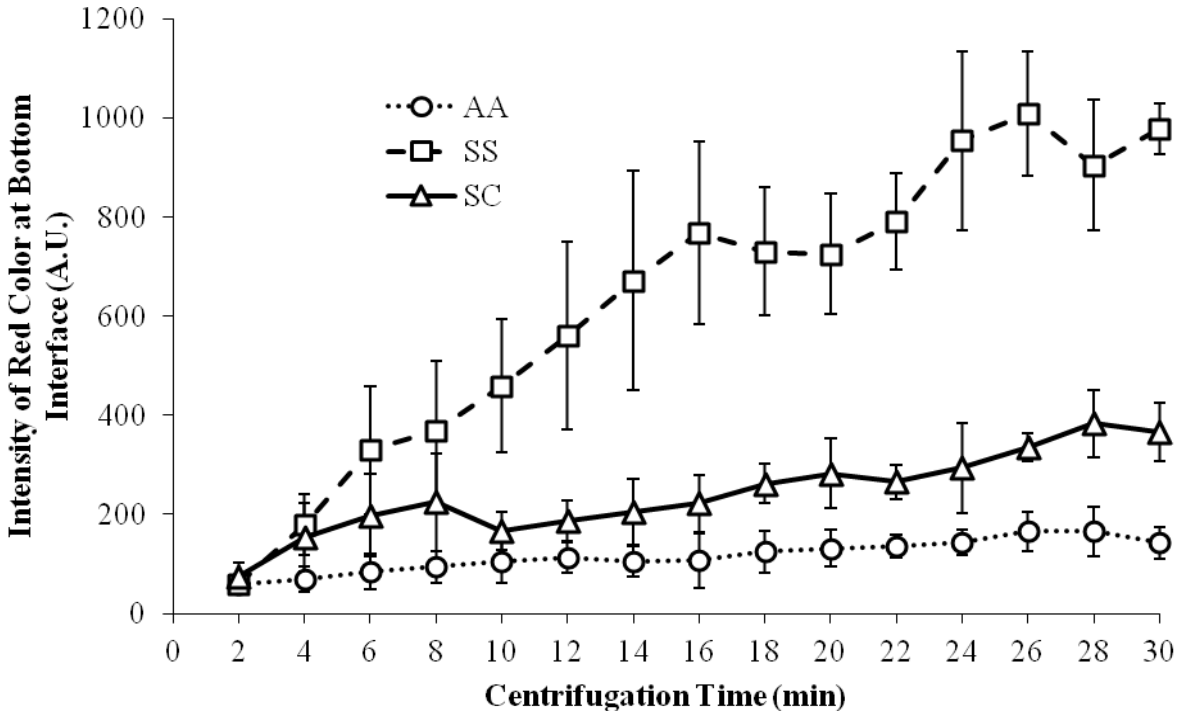
well-defined interface. In a three-phase system, a middle phase with a density of ~ 1.11 g/cm^3 will separate the main population of normal erythrocytes from the high-density tail of the distribution of cells. The middle phase of the three-phase system allows us to distinguish subtypes (**Figure 1**).

Co-solutes tune the osmolality and density of an AMPS to physiological levels. Any swelling or dehydration of erythrocytes that reduces the separation between the three sub-populations of interest may compromise a diagnostic test based on density. To maintain physiological conditions and prevent changes in volume of the cells, we wanted to maintain an osmolality that was isotonic with blood (~295 mOsm) (13).

Achieving the densities necessary to separate dense cells in SCD with polymers alone is difficult. High concentrations of polymer create viscous and hypertonic environments. For example, a solution of 30% (wt/vol) dextran (MW = 500 kDa) in a phosphate buffered solution has a density of 1.122 g/cm^3 and an osmolality of 336 mOsm. This system would dehydrate normal erythrocytes and could increase their density to be indistinguishable from dense cells from SCD. To generate phases with high density that are isotonic with blood, co-solutes with high densities can be used to increase the density of an AMPSs (14).

Separation over Time. In order to choose the time of centrifugation for our rapid test, we performed a time series experiment with the SCD-AMPS-3 system. Six replicates of the rapid test were loaded with blood (n = 2 with Hb SS, n = 3 with Hb SC, and n = 4 with Hb AA). The tests were subjected to centrifugation for two minutes and then scanned in repeated iterations for a total centrifugation time of 30 minutes. The scanned images were then analyzed for the intensity of the red color at the bottom of each test (**Figure S1**). After six minutes, both the Hb SS and Hb SC blood begin to collect significantly more red color at the bottom of the

Figure S1. The intensity of the red color at the bottom interface of the SCD-AMPS-3 system increases with centrifugation time. We evaluated a set of six replicates digitally from samples of Hb AA (AA, n = 4), Hb SS (SS, n = 2), and Hb SC (SC, n = 3) at two minute increments of centrifugation. Error bars depict the average deviation from the mean value of the intensity of the red color from the different subjects. After six minutes, the signal from SCD postive samples (SS and SC) are distinguishable from SCD negative samples (Hb AA). The separation, in general, increases over time. Notably, blood with Hb SS has a significantly higher signal than Hb SC over time.



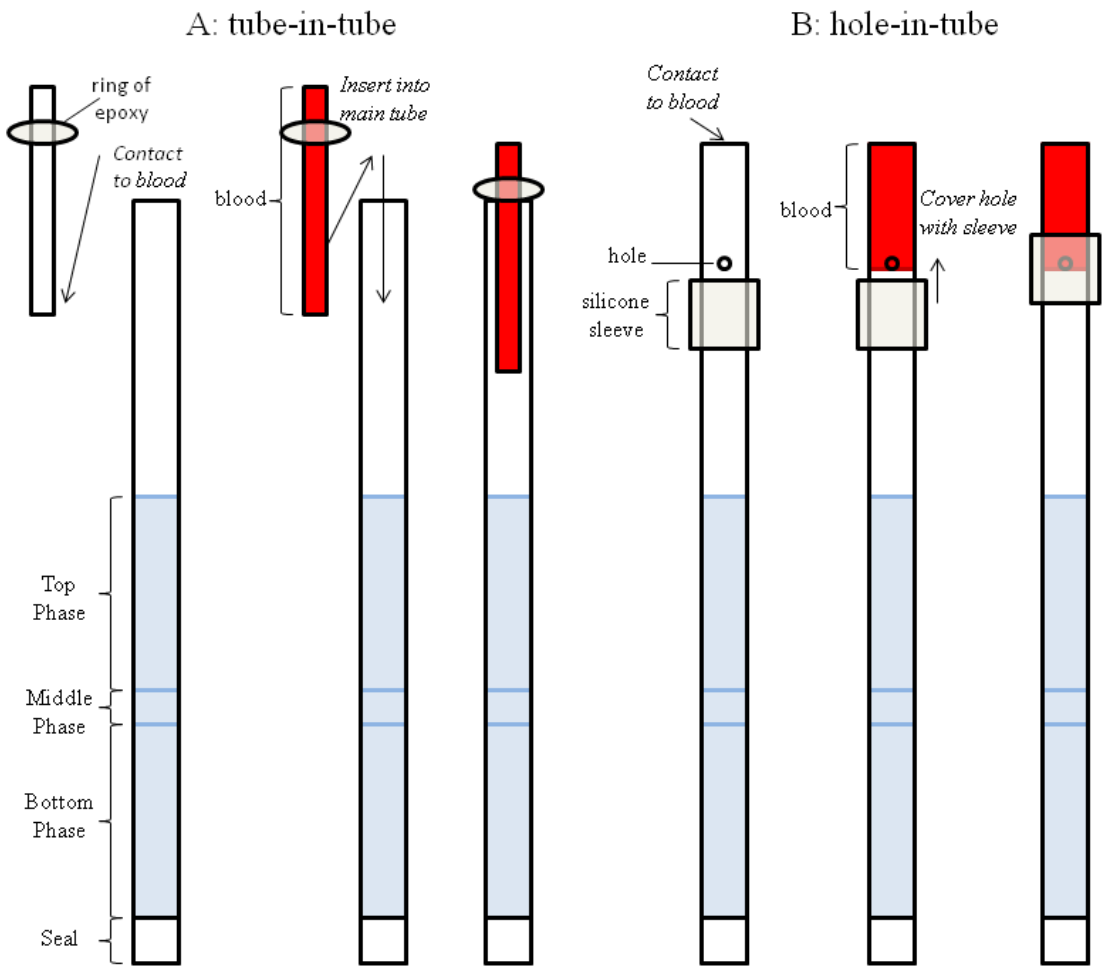
tube than the Hb AA blood. This difference gradually increases over time. To take advantage of this signal amplification without compromising the rapidity of our test, we chose to centrifuge our test for 10 minutes.

Rapid Test Capillary Tube Design. We created two methods to load blood into a sealed tube that was preloaded with an AMPS (**Figure 2**). We have described the "hole-in-tube" method in the main text. Briefly, we use a pushpin in a custom-made alignment mold to puncture the side of the plastic capillary tubes at a specific point along the length of the tube to ensure a repeatable volume is added to all tubes. Air, which would otherwise be trapped and block capillary action, escapes through the hole. To prevent blood from escaping through the puncture during centrifugation, we slid a sleeve of silicone rubber over the hole.

The other method, tube-in-tube, relies on the use of a smaller glass capillary tube that fits within the larger, preloaded polycarbonate capillary (**Figure S2A**). We used the smaller capillary to wick blood into a controlled volume and then introduced the smaller capillary directly into the larger capillary. A small ring of epoxy on the upper portion of the small capillary prevents the small capillary from entering the SCD-AMPS upon centrifugation. This method is fast, but requires some manual dexterity to load the smaller capillary into the larger one.

The tubes hold ~24 μL of liquid in addition to the seal. This provided a constraint to design the volume of our test. Double the volume of blood per test is reserved for loading the sample and then ensuring that once the sample passes into the AMPS, the combined volume is not higher than the hole in the side of the tube—we found that liquid above this level would occasionally leak out, and, if blood was being used, would present a biohazard. Early screening of AMPSs for the sickle test had used a volume ratio of blood to AMPS of 1:3. As we scaled

Figure S2. Two designs to load blood samples into a capillary that has been preloaded with SCD-AMPS-3 and sealed. In the "tube-in-tube" method (A), a small capillary with a ring of epoxy around it fills with blood by capillary action. This small tube can then be loaded into the larger capillary. In the "hole-in-tube" method (B), a small hole allows blood to wick into the prefilled tube. A silicone sleeve prevents the blood from leaking during centrifugation.



down to the rapid test format, we needed to maintain this ratio to maintain a similar performance. Using 14 μL of AMPS and loading 5 μL of blood allowed us to satisfy all our constraints.

Fabrication. We used calibrated micropipettors to fill tubes with the specific volume and then used the fill line to measure the distance we used. We used a custom built hole puncher to make repeatable holes (see *Materials and Methods*). By eye and by pipette the volumes filled were similar. From scans, we estimated the distance between the end of the capillary and the far end of the hole to have a coefficient of variance (CV) of less than 2%.

Hematocrit and Packing of Cells. By comparing the volume that the cells occupied in these three regions to the volume of blood loaded, we can estimate hematocrit (**Figure S3**). We measured the height of the packed cells in each area digitally (ImageJ) and compared it to the length from the hole in the side of the tube to the top of the tube. The low volume of blood used and slight variations in the volumes of the blood and AMPS only allow, however, for a coarse measure of hematocrit (± 10 %). In general, the hematocrit after 10 minutes in the AMPS was an overestimate of the real hematocrit. Additional centrifugation time improved the packing of the cells (**Figure S4**) and could improve the hematocrit estimation.

Aggregates in Negative Samples. To investigate the white or pink layer that occasionally formed at the bottom of negative samples, we examined the material by optical microscopy. We identified the samples to investigate by using the smaller rapid test format, but we could not extract enough material from these systems to identify the objects under a microscope. We, thus, scaled up the separation to a 1.5 mL Eppendorf tube, while maintaining the same ratio of blood to AMPS and comparable centrifugation parameters. After separation, we used a micropipettor to extract the layer of material below the bottom phase of the AMPS and stained a thin smear of the sample on a glass slide **(Figure S5).**

Figure S3. The hematocrit measured in AMPSs provides an estimate of the spun hematocrit. A range of hematocrits was made by mixing packed cells with homologous plasma. The estimated hematocrit from AMPS is generally a slight overestimate of the real hematocrit because at 10 minutes, the cells are not completely packed. The dashed line indicates the line of equivalent values.

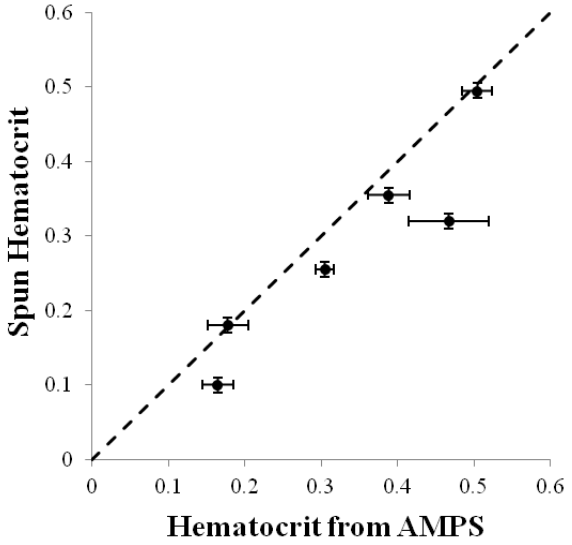


Figure S4. Additional centrifugation time results in clearer separation of the cells. Some isodense cells remain unchanged after 30 minutes (Hb SS). Hb SC is easily distinguished from Hb SS after 20 minutes. The pack of cells above the white clay seal (dark gray in transmission imaging) increases over time (Hb SS and Hb SC).

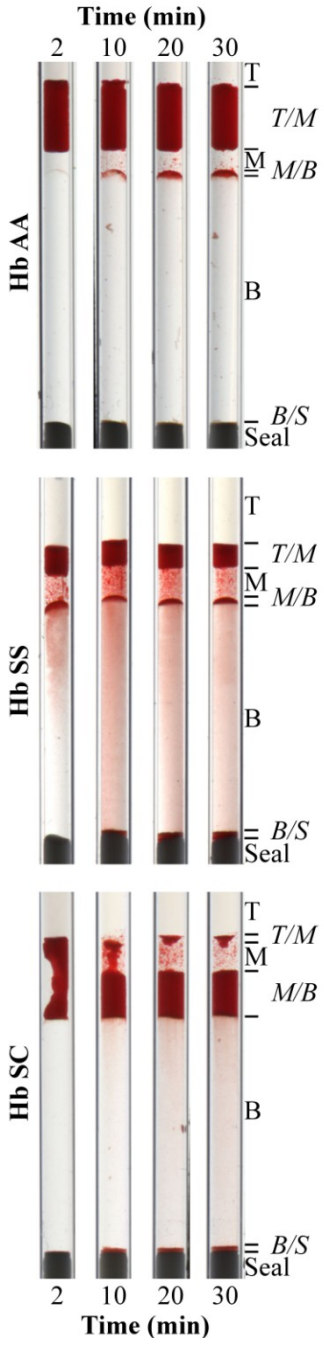
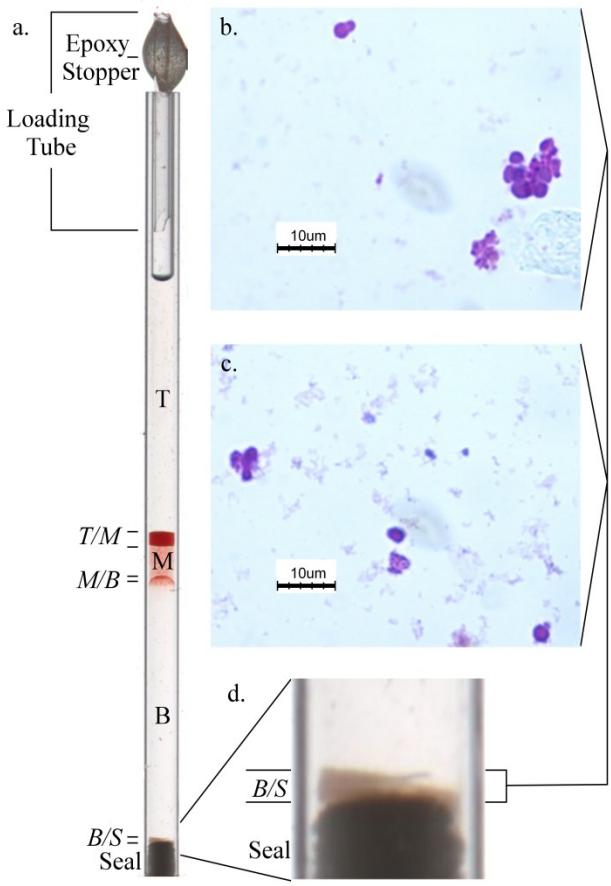


Figure S5. Example of white pellet found at the bottom of some of the samples from Hb AA subjects. A tube-in-tube version of the SCD-AMPS-3 test is negative for SCD (a.). Although the bottom is not red, there is a substantial gray layer above the white clay seal (dark gray in transmission imaging) (*B/S*) (d.). Micrographs (b. and c.) reveal a large number of platelets and cell aggregates.

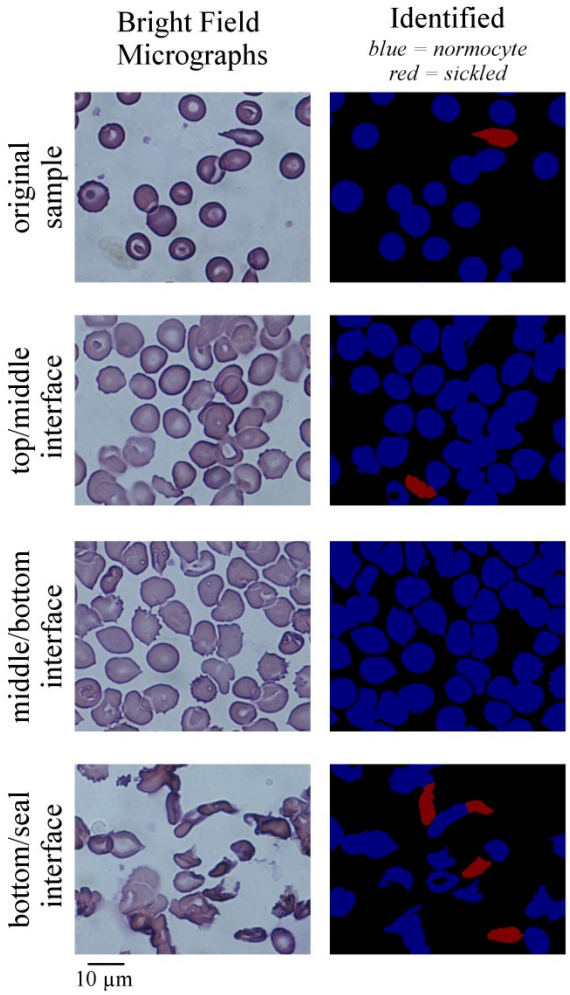


After centrifugation, we extracted and washed the cells from each interface of the AMPS. We made thin smears stained with Hemacolor (Harelco) to evaluate the morphological distribution of erythrocytes.

Using bright field microscopy we captured a series of images, which we then analyzed with CellProfiler™ (Broad Institute) to quantify the percentage of erythrocytes that were sickled in each interface. We classified a cell as sickled if the aspect ratio of the major axis length over the minor axis length was greater than 2. We found this measure to correlate well with sickled morphologies over several fields of view that we evaluated by eye (**Figure S6**).

When using blood from a patient with Hb SS with a very low level of sickled cells (0.7%), we were able to visualize the presence of dense cells at the bottom of the SCD-AMPS in both the capillary tubes and the microcentrifuge tubes. The fraction of cells at the bottom phase/seal interface contained 7.3% sickled cells. Over half of the remaining cells appeared crenated and dehydrated, similar to the “holly wreaths” or "holly leaf shapes" that result from deoxygenation of cells with Hb SS (15–17). Upon entering the SCD-AMPS, the erythrocytes may have deoxygenated. Rapid deoxygenation in Hb SS causes the formation of crenated cells and “holly-wreaths” or "holly leaf shapes" instead of the classic sickle shape (15–17). Normal erythrocytes in the smears from the bottom fraction may be either cells that have been oxygenated during the washing step and returned to a normal morphology or normal cells that became engulfed by a mass of dense cells and trapped at the bottom of the tube. Interestingly, the cells at the upper and lower liquid/liquid interfaces had 4.4% and 4.7% sickled cells, respectively. These layers, however, did not contain crenated, dehydrated cells. The existence of cells with a high aspect ratio may have been a result of smearing cells from the polymer

Figure S6. Micrographs of blood from the different fractions of a sample with Hb SS run on SCD-AMPS-3 test is evaluated digitally to quantify sickling. Identified cells are classified as normocytes (blue) or sickled (red). The cells at the bottom interface (bottom/seal) are markedly more sickled and dehydrated.



solutions, but the higher proportion of cells with high aspect ratios in the bottom layer suggests that there was a higher amount of sickled cells in the bottom population.

Quantification of Dense Cells. To quantify the percentage of dense cells, we evaluated the digital images of the results from the SCD-AMPS-2 tests. Using digital analysis (ImageJ) we measured the height of the packed cells above the seal and the height of the packed cells at the liquid interface. We then calculated the percentage of dense cells for all the samples that had SCD (both Hb SS and Hb SC) (**Table S3**). Note that in two cases of Hb SS, we did not visually identify a band of red cells at the bottom and the calculated percentage of dense cells in these cases was zero. Of the 21 SCD samples that were tested on SCD-AMPS-2, the average percentage of dense cells was 10%.

Results by Genotype. The sensitivity and specificity values described in the main text were based on binning all positives (Hb SS and Hb SC) together and all negatives (Hb AA and Hb AS) together. **Table S4** details the results of visual evaluation of all four genotypes in this study. Five of the six Hb SC samples could be distinguished from Hb SS after evaluating the distribution of cells between the two liquid interfaces in SCD-AMPS-3. All Hb SS samples appeared as expected in **Figure 1;** none of the Hb SS samples appeared with a majority of the red cells at the lower liquid interface.

Nystatin provides a means to create model SCD blood. Testing the diagnostic capabilities of the SCD-AMPS required samples of blood from SCD patients that had not been recently transfused (transfusion reduces the number of dense cells present in a patient's blood). To characterize the limit of detection of our system in a quantitative way we needed model dense cells whose behavior was less subject to change than sickle cells, we created dense erythrocytes

Table S3. Quantification of the dense cells from the SCD-AMPS-2.

Donor	Genotype	Visual Reading	Dense Cells
1	SS	Positive	21%
2	SS	Positive	15%
3	SS	Positive	8%
4	SS	Positive	16%
5	SS	Positive	14%
6	SS	Positive	10%
7	SS	Positive	12%
8	SS	Positive	12%
9	SS	Positive	15%
10	SS	Positive	11%
11	SS	Positive	8%
12	SS	Positive	13%
13	SS	Positive	10%
14	SS	Negative	0%
15	SS	Negative	0%
16	SC	Positive	8%
17	SC	Positive	8%
18	SC	Positive	10%
19	SC	Positive	4%
20	SC	Positive	9%
21	SC	Positive	8%
Average	--	--	*10%*

Table S4. Visual evaluation of the SCD-AMPS for sample sizes of *N*.

	SCD-AMPS-3			SCD-AMPS-2		
Sample	***N***	**Positive Rate[a]**	**Negative Rate**	***N***	**Positive Rate[a]**	**Negative Rate**
Hb SS	20	**0.90**	0.10	15	**0.87**	0.13
Hb SC	6	**1.00**	0.00	6	**1.00**	0.00
Hb AA	26	0.15	**0.85**	24	0.04	**0.96**
Hb AS	7	0.00	**1.00**	7	0.00	**1.00**

[a] Rates were calculated by comparing the results from the AMPS test to the known status of the subjects as measured by a gold standard (either HE or HPLC).

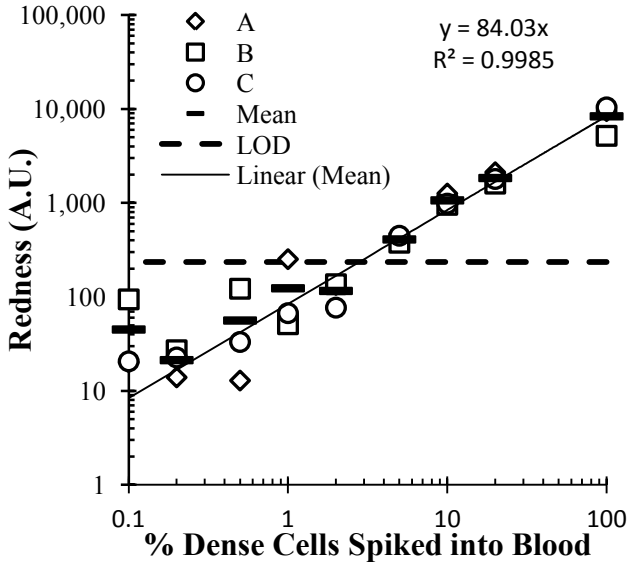
by treating blood with nystatin, and exposing them to hypertonic media (12). This creates dense, dehydrated cells (18); we used these cells as a model of dense cells.

We mixed known volumes of these dense cells with untreated blood to simulate SCD blood. The model blood contained small (cell volume < 60 fL) cells (microcytic) with high concentrations of hemoglobin (hyperchromic), similar to sickled cells in SCD; after treatment, the erythrocytes from three blood samples had a mean corpuscular volume ranging from 67.3–71.5 fL, and mean corpuscular hemoglobin content ranging from 39.9–41.5 g/dL. When we mixed 5% of the treated cells with the original blood, the model samples had a mean corpuscular volume ranging from 81.9–83.9 fL, a mean corpuscular hemoglobin content ranging from 32.4–34.8 g/dL, and the percent of microcytic erythrocytes ranged from 2.6–3.8%.

Determination of the Limit of Detection Using a Model System for SCD. Using normal blood (n = 3) spiked with dense cells created by the nystatin treatment, we evaluated the bottom of the SCD-AMPS-3 for the presence of red color after 10 minutes of centrifugation. By eye, we could detect the presence of dense cells in normal blood at a concentration of 2% about half the time. At a concentration of 5%, a layer of red covered the bottom of the capillary. Most SCD patients have over 13% dense, SCD cells.(3)

We also imaged the results of each test with a flatbed scanner in transmission mode (Perfection Photo V550, Epson). Image processing in Matlab evaluated the amount of red that had collected at the bottom of each capillary. **Figure S7** depicts the measured value of the "intensity of red color" in arbitrary units (AU) for the different concentrations of dense cells that were added to the normal blood. We found a good linear fit ($R^2 > 0.995$) to the data with an intercept set at 0. For the digital analysis, we found the limit of detection to be 2.8% dense cells by finding the value of the linear fit that provided a signal that was three standard deviations

Figure S7. Measuring the intensity of red color at the bottom of the SCD-AMPS-3 can detect dense erythrocytes in whole blood at a concentration of 5%. Erythrocytes from three donors (A–C) were treated with nystatin to be dense and dehydrated, and then spiked into untreated blood at known concentrations. After centrifugation in a tube containing the SCD-AMPS-3, the tests were scanned and analyzed to quantify the presence of dark red bands below the bottom phase. The limit of detection (dashed line) was established as three standard deviations above the mean measured on normal blood (n = 7).



above the signal from normal blood (n = 7). Below this concentration, it is possible for the digital analysis to confuse results from normal blood and SCD blood; this limit provides the false positives and false negatives that were observed in **Figure 4**.

Alternative Methods to Diagnose SCD at the Point-of-Care. Miligan *et al.* have proposed monitoring hemolysis in non-electrolyte solutions as a means to diagnose sickle-cell disease (19). Quantifying hemolysis allows them to distinguish some genotypes and may provide a means to monitor certain clinical effects of SCD (**Table S5**). This test requires an hour of incubation, the use of an expensive tonometer, and optical density measurements; meeting these requirements in a point-of-care setting may be challenging.

The recent development of a paper-based test for SCD may provide an alternative low-cost diagnostic test (20). This test distinguishes Hb AA, Hb AS, and Hb SS visually by evaluating blood stains on paper after lysing and deoxygenating the hemoglobin (using a method similar to a solubility test). The visual signal can be analyzed by a scanner and correlates to the concentration of Hb S present. Even with the use of the digital analysis, the test is, however, less accurate than the AMPS-based tests at distinguishing individuals with Hb AS (non-disease) and Hb SC (disease); the Hb S concentration in these two genotypes can be very similar. In a person with Hb SC, the presence of Hb C leads to dehydration that induces sickling at a significant level that would not take place in a person with similar levels of Hb S, but with Hb AS (21).

Distinguishing between Hb SC and Hb AS is clinically important, especially in West Africa where both genes are common. In settings where Hb C is rare, such as eastern and southern Africa, this test could be a quick and inexpensive way to identify and distinguish between sickle cell trait and disease.

Table S5. Comparison of methods to detect SCD.

Ref.	Method	Time (min)	Differentiation				Fieldable	Biophysical Indicator	Sample Prep. Free	Instrument Free	Instrument Cost	Unit Cost
			AS /SS	AS /SC	SC /SS	AA /AS						
This work	AMPS	12	✓	✓	✓[a]		✓	✓	✓		$150-1,600	$0.50
(19)	Hemolysis	> 60	✓		✓			✓[b]			~$10,000	NA
(20)	Paper	20	✓	✓[c]	✓	✓	✓			✓	$300-500	$0.07[d]
†	Solubility	5				✓	✓			✓	$0	$3.00
†	HPLC*	> 120	✓	✓	✓	✓					>$60,000	$10.00
†	HE*	> 180	✓	✓	✓	✓					>$10,000	$3.00
†	Genetic	>180	✓	✓	✓	✓					>$20,000	$1.00

[a] specifically, SCD-AMPS-3

[b] under investigation

[c] except in cases where hemoglobin S levels are close

[d] based on cost estimates for a similar paper test (22)

† based on market prices and product literature

* gold standard method

Conventional techniques of separation by density are not suitable for use in field settings. Sequentially layering solutions with decreasing concentrations of a dense solute (e.g., sucrose, Percoll, arabinogalactan) creates a layered gradient in density (23). These gradients can separate blood into multiple subpopulations of cells of different densities. Layered gradients in density are not practical for use in a point-of-care test for several reasons: i) diffusion-driven homogenization of layers limits the long-term storage of a layered gradient, ii) agitation or mixing destroys a layered gradient, and iii) assembly of a gradient requires careful and tedious layering, and a high level of technical competence.

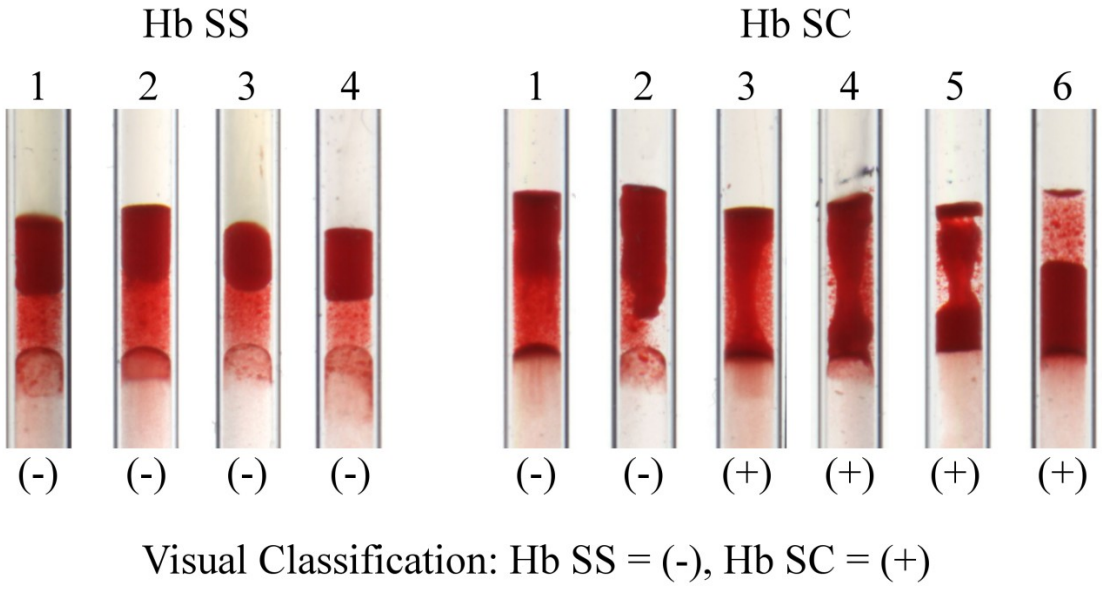
Centrifugation of blood over mixtures of phthalate esters provides a simpler method to characterize the density profile of blood (24, 25). Mixtures of phthalate esters provide a range of single-density media. Upon centrifugation, blood cells either sink or float in a phthalate solution based on the difference in density between the cells and the solution. The immiscibility of the phthalates and water ensures that cells at the top of the phthalate are packed at an interface (i.e., a water/phthalate interface); cells layered over an aqueous medium would collect in a diffuse boundary between the plasma and the medium. Packing cells is an important characteristic for a separation to provide quantitative information. Comparison of the volume of packed cells above and below a phthalate provides a measure of the distribution of the density of cells. Although simpler to use than layered gradients, phthalate esters are unsuitable for point-of-care use for two reasons: i) they require a temperature-controlled centrifuge (26), and ii) they cannot distinguish more than two subpopulations of cells in a single system—a necessary ability to differentiate sub-types of SCD by density.

AMPSs combines the best aspects of layered gradients and phthalate esters, while overcoming the principal drawbacks of each. Like layered gradients, AMPSs allow multiple

sub-populations to be separated in a single system. Like phthalate esters, AMPSs concentrate cells at well-defined interfaces and are easy to use. Together, these characteristics allow centrifugation through AMPSs to distinguish blood from patients with SCD from normal blood by density and classify the two main subtypes of the disease.

Identifying subtypes of SCD. SCD-AMPS-3 is further able to distinguish between the two main forms of SCD: i) Hb SS, which accounts for the majority (~ 75%) of SCD (27), and ii) Hb SC, which constitutes most of the remaining cases of SCD (~20%) (27). These two variants of SCD have important differences in pathophysiology; Hb SC is generally less severe, but is associated with a higher risk for retinal vascular damage and otological disorders, whereas Hb SS is generally more severe and comes with a higher risk of stroke and acute chest syndrome (28–30). Effective diagnosis of the genotype would enable management to be tailored to the appropriate risks. Four of the six samples with Hb SC were classified correctly by visual evaluation after 10 minutes of centrifugation (**Figure S8**). Five of six samples were classified correctly after 20 minutes of centrifugation.

Figure S8. Examples of the patterns of red cells at the liquid interfaces for Hb SC and Hb SS in the SCD-AMPS-3 system. Four representative examples of the layers of red blood cells from samples with Hb SS show the characteristic pattern of the majority of cells packed at the upper liquid/liquid interface with a thin packed band at the lower liquid/liquid interface. All six samples with Hb SC are shown after 10 minutes of centrifugation. Samples Hb SC-4, 5, and 6 all have red bands at the lower liquid interface that are comparable to or greater than the bands at the upper liquid interface. Sample Hb SC-3 has a significant pack of cells at the lower liquid interface an hour glass shape of red cells between the two liquid interfaces. Sample Hb SC-2 packed to a pattern more similar to Hb SC-3 and 4 after 20 total minutes of centrifugation.



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