Supporting Information

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SI Material and Methods

Antibody Conjugates Preparation. All antibody-fluorescence signal conjugation was prepared using a Lightening-Link Conjugation Kit (Innova Bioscience Ltd.), which allows the conjugation of fluorescence tags to antibodies in about 3 h without desalting or dialysis steps. For ferritin, 600 μg monoclonal antihuman ferritin IgG produced in mouse (Scripps Laboratory Inc.) and 60 μL LL-modifier reagent (Innova Bioscience Ltd.) were mixed and diluted to 1 mg/mL before conjugation in 0.01 M amine-free PBS buffer at pH 7.4, and then 0.6 mg RPE was resuspended in the solution. The other two conjugations are prepared with a similar method. For the RBP test, 1.5 mg mouse monoclonal antihuman RBP IgG (HyTest Ltd.) was conjugated with 2 mg FITC, while for the CRP test, 0.6 mg mouse monoclonal antihuman RBP IgG (Biobyt Ltd.) was conjugated with 0.6 mg PE/Cy5. All conjugations are stored at 4 °C in a light-free environment until use, and no obvious degradation is observed after storage as long as 2 mo. Before application to the reaction pad, the conjugation was mixed and diluted to the proper concentration. RPE–anti-ferritin, FITC–anti-RBP, and PE/Cy5–anti-CRP were respectively diluted 200 times, 20 times, and 10 times in the conjugate buffer (2 mM borate buffer with 5% sucrose). On each test strip, 5 μL conjugation antibodies was applied.

Multicolor Fluorescence Lateral Flow Assay Preparation. Test strips are based on High Flow Plus 180 Membrane Cards (HF180; EMD Millipore). Polyclonal goat anti-human ferritin antibodies (Scripps Laboratory Inc.), human RBP4 (HyTest Ltd.), and polyclonal goat anti-human CRP antibodies (CalBioreagents, Inc.) and were diluted to 0.75 mg/mL, 2 mg/mL, and 1.8 mg/mL, respectively, in 1× PBS and dispensed as test lines on the nitrocellulose membrane, as well as 0.40 mg/mL anti-mouse IgG produced in goat (Sigma-Aldrich Co. LLC) as a control line. Subsequently the membrane cards were dried for 2 h at 37 °C to immobilize antibodies on them. To assemble the assay, FR-1 Membrane (035; MDI Membrane Technologies) and Fusion 5 (General Electric Company) were attached as the mixing pad and the reaction pad, while two Cellulose Fiber Sample Pads (EMD Millipore) were attached at both ends to serve as the sample pad and the waste pad of the assay. To ensure consistent flow between pads, all of the pads had an overlap of 0.2 mm at the junction. The assembled assay cards were cut into 4-mm-wide test strips, on which 5 μL conjugation was applied to the conjugation pad, and dried for 1 h at 37 °C in the dark. Then the test strips were sealed and stored at room temperature in the dark until use.

Sample Preparation and Operation Protocol. The whole blood samples were from 43 different adults in the United States. All human whole blood samples were obtained from a commercial provider (Research Blood Components, LLC). The donors are in generally good health status, with no infectious disease according to their self-reports. And all of the blood samples have been prescreened for HIV, hepatitis B, and hepatitis C negative. Serum samples were separated from whole blood with a portable minicentrifuge (ChemGlass) at 2,000 × *g* for 10 min before tests were performed. Then 15 μL of serum sample was applied on a reaction pad immediately after separation. The test strip was then incubated in the dark for 2 min, followed by adding 60 μL of running buffer (pH 6 buffer from Sigma-Aldrich, diluted to 50% in deionized water, with 1× Tris-buffered saline, 0.5% Tween 20, and 1% BSA) to initialize the test. The TIDBIT reader automatically captures and analyzes the fluorescence image after 15 min and sends results to the connected interface device.

SI Results

Test Length Optimization. Accurate, timely, and quantitative readout is an important feature of point-of-care assays. In many cases there can be a tradeoff between reducing the time to the result and overall accuracy of the test. To analyze this here, we ran a series of dynamic test-line development assays. To avoid variation caused by serum biomarker characterization, we resuspended linear dried serum to the desired known concentrations and applied the sample to the test strips. The TIDBIT reader was also reprogrammed to take repeated fluorescence images every 90 s and save the image acquisition for further analysis. Fig. S1*A* shows the image set for a sample with ferritin, RBP, and CRP concentrations at 217 ng/mL, 29.7 μg/mL, and 3.3 μg/mL, respectively. It shows clearly that intensity of test lines increased while the sample moved along the flow at first, and running buffer washed away unbound antibody conjugation later.

The dynamics test results for three biomarkers are shown in Fig. S1 *B*–*D*. The rates of test line intensity development for ferritin and CRP were positively correlated with the concentration. CRP test lines developed to their maximum earlier than ferritin because they were farther upstream. At low RBP, test line intensity was shown to decrease from $t = 270$ s to $t = 450$ s, which suggests that RBP molecules in the sample were competing with RBP molecules on the test line. Overall, however, the result indicates that all test line intensities remained stable from $t = 810$ s to t = 1,200 s. As a result, the TIDBIT system captures the fluorescence image at $t = 900$ s to ensure full development of the test lines. The dynamics of test line intensities also prove that photobleaching of fluorescence signal caused by excitation LEDs in the TIDBIT system during the test was negligible.

Microcontroller Software Function Design. To enable wireless control of the TIDBIT reader, the single-board computer was configured as a web server. When the device was turned on, it broadcasted a wireless network, which was detected by any mobile device with WiFi connectivity. Once the mobile device was connected to the network, commands were sent to and from the reader via HTTP request. The image-processing software on Raspberry Pi was written in Python. OpenCV was used to read, write, and crop images. No other major third-party libraries were used. When the TIDBIT reader captured an image, all electronic autosettings in the camera were turned off to ensure measurement accuracy. The exposure time was set to 10 s for the camera to receive enough light. After capturing the image, the device outputted a correlated image with ∼15 MB raw image data appended to it. As shown in Fig. S1, the appended raw image data were in RGGB Bayer format, and the nearest-neighbor interpolation demosaicking algorithm was applied. After the reforming of the raw image was completed, a series of image-processing steps were performed to quantitatively analyze the image. Then the image was cropped with mathematical morphology operations, so that only the test-strip area remained.

Background Removal and Image-Processing Algorithms. Based on the mechanics of the immunoassay, the number of fluorophore molecules captured by the test line should be proportional to the number of analyte molecules in a sandwich-type assay or the number of unoccupied conjugation antibody molecules in a competitive assay. As a result, the software captures the total

fluorescence emission throughout the entire capture site. To do this, the software measures the fluorescence signal within a rectangle, which covers all pixels through the width of the test strip and the widest point on the fluorescence band. In detail, the software first reduces the 2D image to a 1D array by simply taking the total brightness value of each pixel through the width of the test strip. The background signal in the 1D array was then removed by subtracting the four-order polynomial fitting curve of the points on the background, as described in Fig. S3. After the background is subtracted, the software recognizes each test band and finds where the start and end points are within the 1D array. This represents the location of the widest bandwidth in the original image along the test-strip length. By adding all of the values between the start and end points in the 1D array, the total fluorescence emission signal output is obtained. Moreover, variation caused by strip-to-strip bandwidth difference can be reduced through normalization of the total fluorescence signal output by the area of the fluorescence band.

Finally the average brightness values of each test line were converted into ferritin, CRP, and RBP concentrations based on the [marker] = f[brightness #] curve, which was predetermined with calibration data. The data points are fitted to intensity of signal = d + (a–d)/(1+([marker]/c)^b) with MedCalc, and the values of the parameters are shown in Table S1.

Range of Biomarkers for Quantitative Analysis. The device provides quantitative analysis for all three biomarkers (ferritin, RBP, and CRP) within a physiologically relevant dynamic range for the given marker. These ranges are as follows: (*i*) RBP, 2.2–20 μg/mL (0.10–0.95 μmol/L); (*ii*) ferritin, 12–200 ng/mL (26–421 pmol/L); and (*iii*) CRP, 0.5–10 μg/mL (20–400 nmol/L).

If the concentration of a given marker falls within the physiologically relevant dynamic range, the TIDBIT reader provides a quantitative result for all three biomarkers. Otherwise it tells whether the concentration of the given biomarker is greater than the upper limit of quantification or less than the lower limit of quantification. The lower bound on the dynamic range is governed by the limit of detection of the TIDBIT, while the upper bound is limited by the natural mechanics of the sandwich and competitive assay.

To be more specific, as we explained in *Cross-Binding and Limit of Detection Quantification*, for CRP the quantitative range is between 0.5 μg/mL and 10 μg/mL, which spans the physiological range widely used to determine inflammation status. Between these ranges the device returns the quantitative value of the measured CRP. Below this range we just present "<0.5 μg/mL," indicative of a low level of inflammation and approaching the limit of quantification for the device. For RBP the range of quantification is between 2.2 μg/mL and 20 μg/mL. As above, we therefore present a quantitative number between this range and "<2.2 μg/mL" below the lower limit of quantification (although no samples in the study had such a low level of RBP) and ">20 μg/mL" above this range. For ferritin we found the test has a lower limit of quantification at 12 ng/mL. As a result, if the ferritin concentration is lower than 12 ng/mL, the system returns the result "<12 ng/mL."

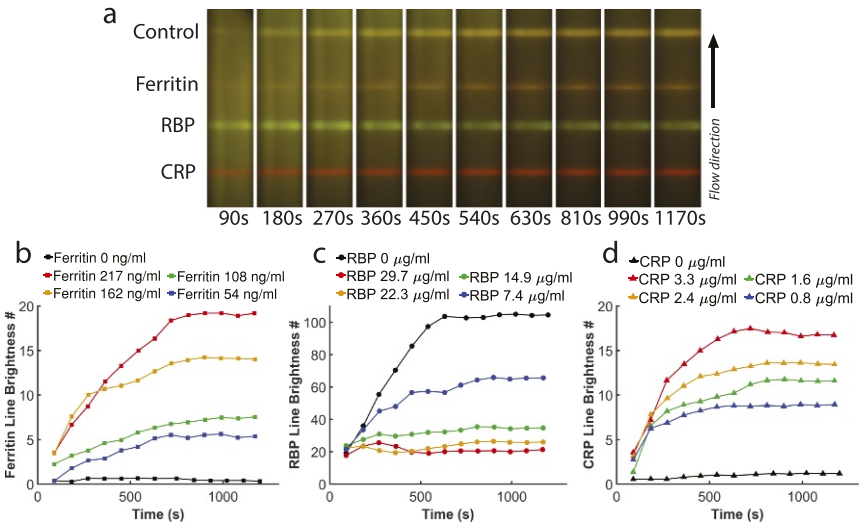

Fig. S1. Dynamics of the test lines intensity. (*A*) Fluorescence image series for dried serum samples with 217 ng/mL ferritin, 29.7 μg/mL RBP, and 3.3 μg/mL CRP. Images were captures every 90 s. (*B–D*) Dynamics of test line intensity in multiplex tests. Five (red, orange, green, blue, and black lines) resuspended dried serum samples were used. They show that test line intensity remains stable after *t* = 00–1,200 s.

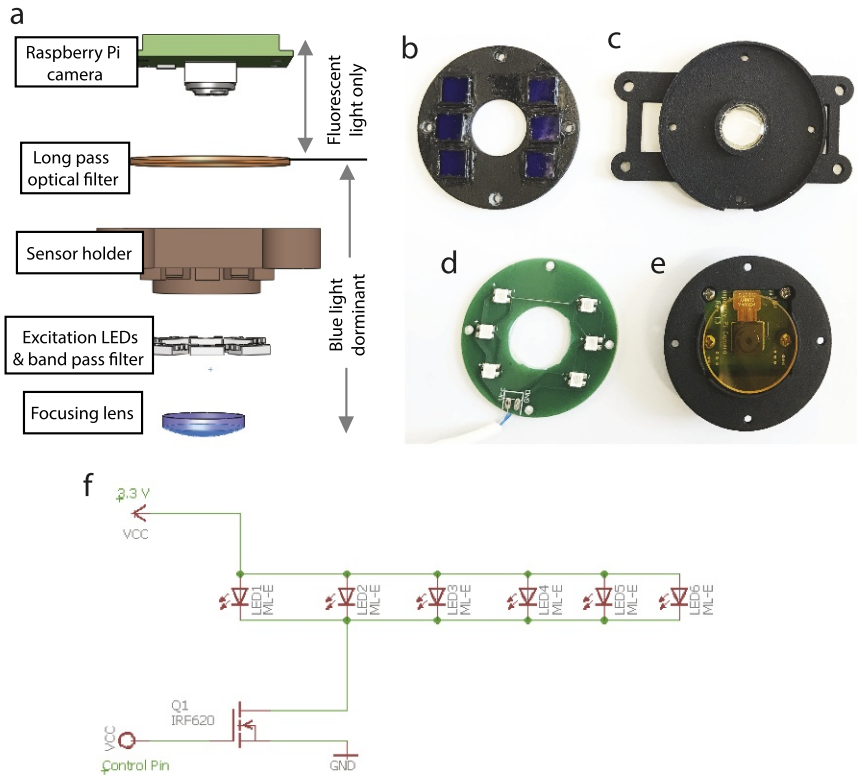

Fig. S2. Fluorescence sensor parts and diagrams. (*A*) Fluorescence sensor optical diagram. The sensor excites the fluorescence signal with LEDs and captures the image at the same time. (*B*) Excitation optical filter. (*C*) Focusing lens holder. (*D*) LED on printed circuit board. (*E*) Camera and emission optical filter. (*F*) Electronic part diagram.

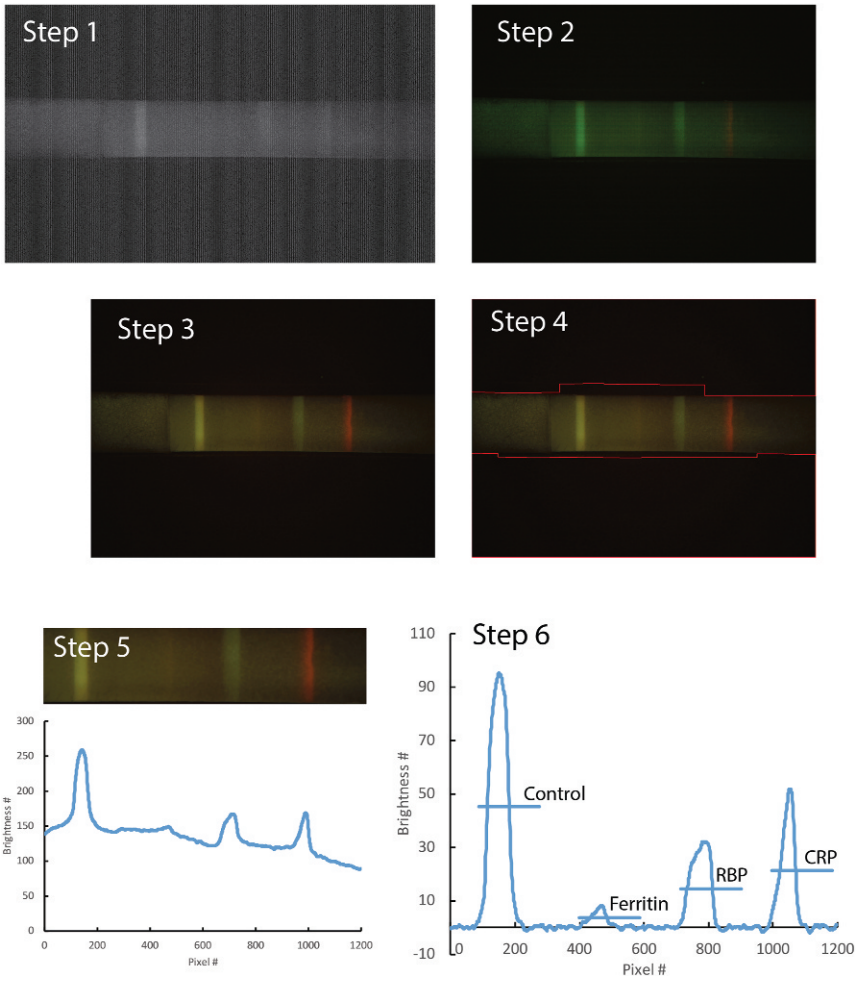

Fig. S3. Image-processing example. Step 1, Raw data are acquired from camera CMOS sensor; step 2, convert raw data to Bayer pattern image; step 3, Bayer pattern demosaicking, where output image shows true intensity of fluorescence signal; step 4, autoshape recognition to determine test-strip area in image, and background is cropped; step 5, add brightness value along each row to reduce 2D image to 1D array; and step 6, reduce background noise in 1D array and find peak average as brightness number. The brightness numbers are then used to calculate ferritin, RBP, and CRP levels according to the logistics fitting curve.

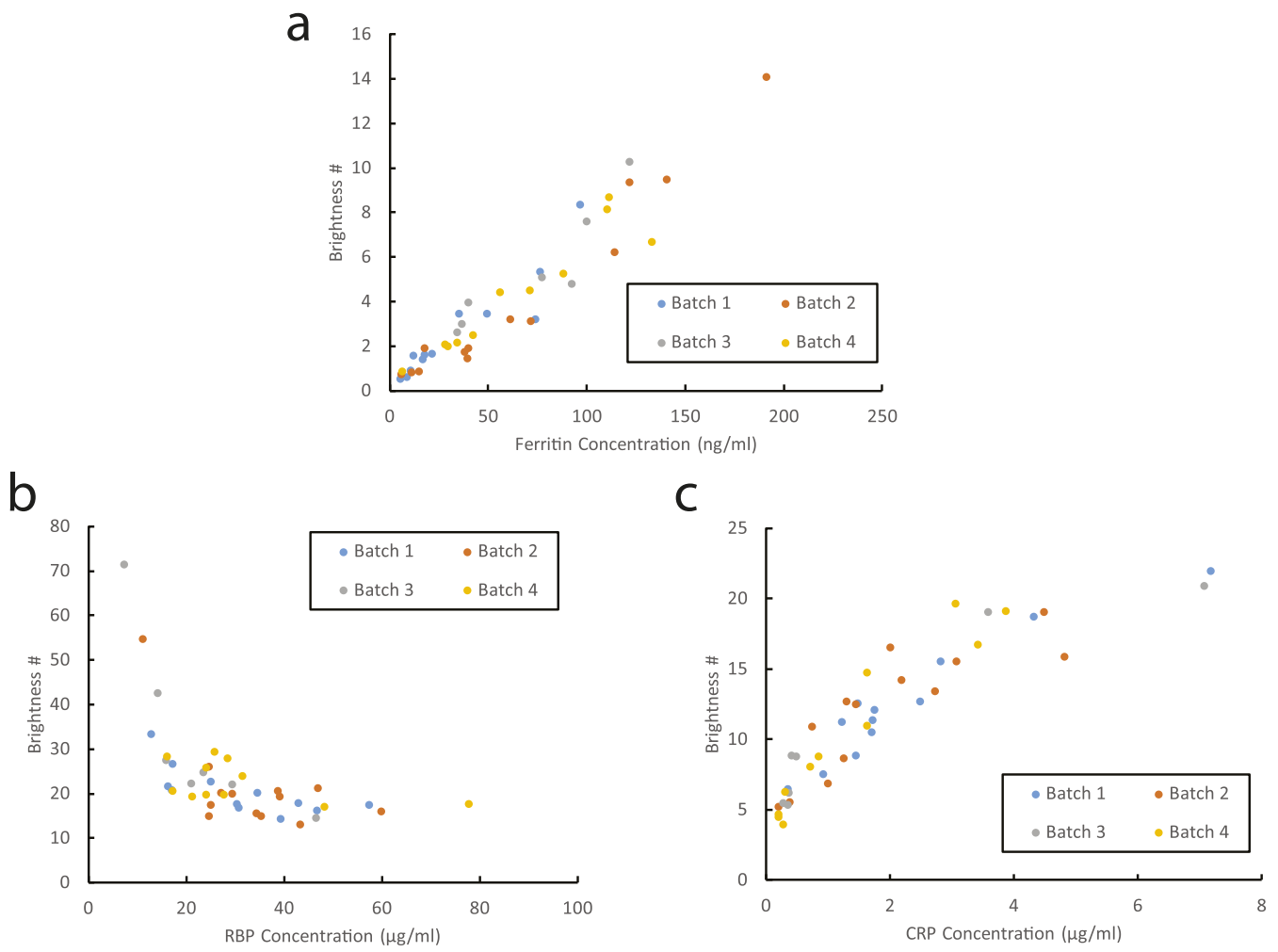

Fig. S4. Batch-to-batch differences comparison. Four batches of test strips were manufactured with the same reagent and used to test the 43 human samples. Shown is the batch-to-batch result comparison for (*A*) ferritin, (*B*) RBP, and (*C*) CRP. No significant differences were observed between test-strip batches.

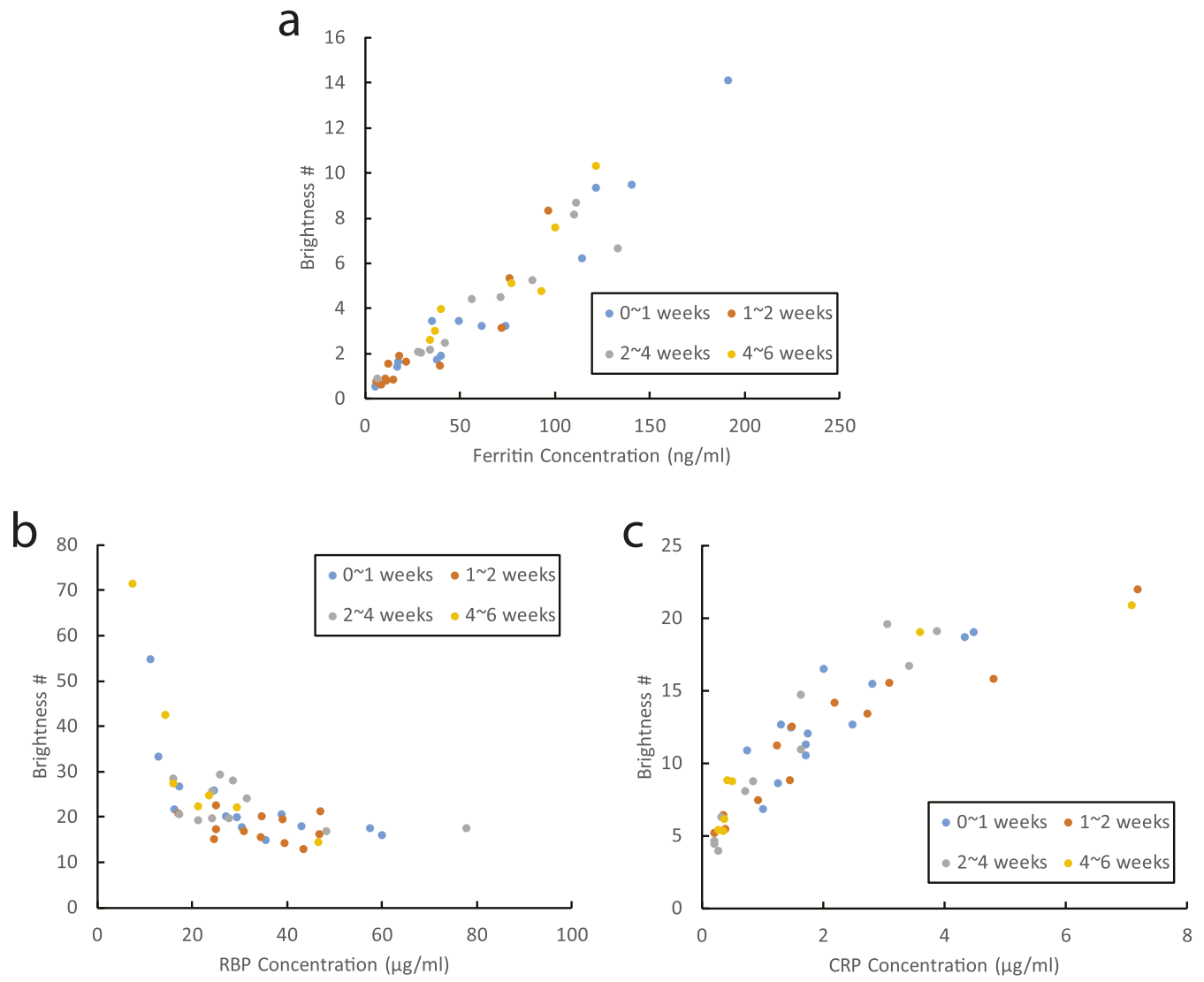

Fig. S5. Effect of storage time on test results. Shown is a comparison of test results that been stored for different lengths of time for (*A*) ferritin, (*B*) RBP, and (*C*) CRP. No significant differences were observed within 6 wk of storage.

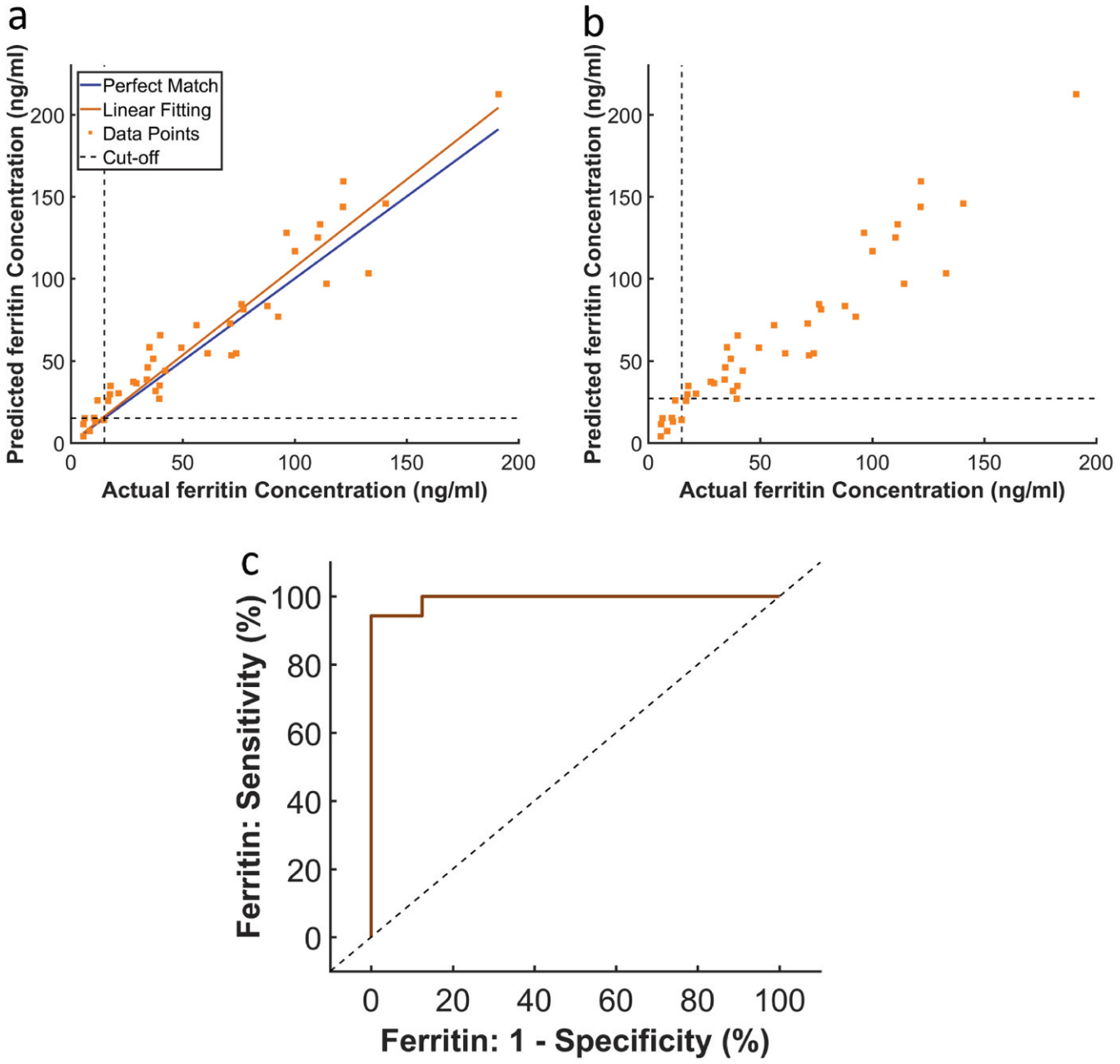

Fig. S6. (*A*) Comparison between actual and predicted concentration for ferritin. Orange line indicates RC = 1.06. Dashed line indicates cutoff at 15 ng/mL (32 pmol/L). (*B*) Comparison between predicted and actual concentration for ferritin and cutoff at 27 ng/mL, when the system yields sensitivity of 100% and specificity of 94%. (*C*) ROC curve for ferritin.

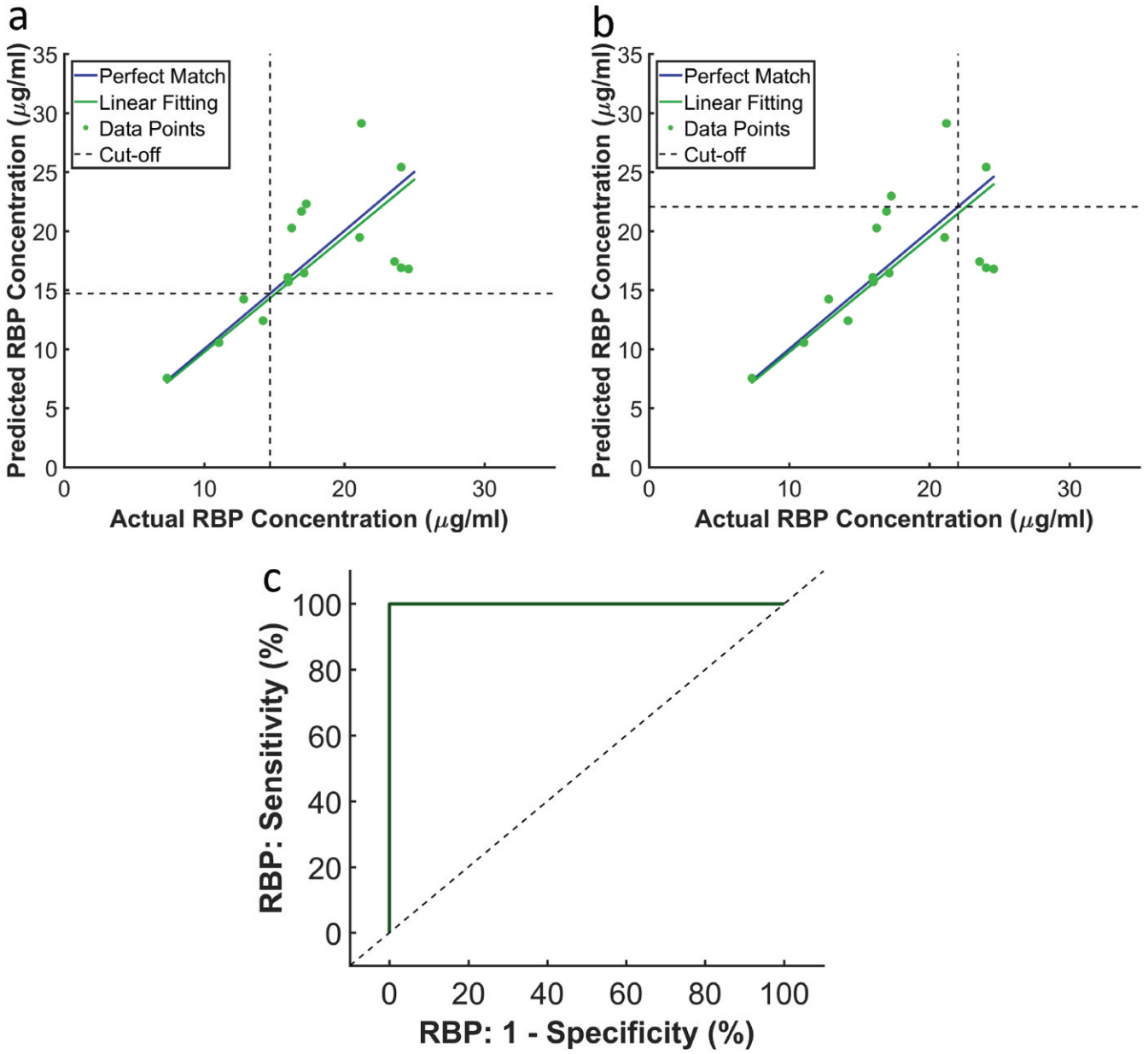

Fig. S7. (*A*) Comparison between actual and predicted concentration for RBP. Since the assay gives only a quantitative result for RBP <25 μg/mL, only sample RBP <25 μg/mL was compared. Green line indicates RC = 0.97. Dashed line indicates cutoff at 14.7 μg/mL (0.70 μmol/L). (*B*) Comparison between actual and predicted concentration for RBP, with cutoff at 22 μg/mL (1.05 μmol/L). For the midrange (∼15–22 μg/mL or ∼0.70–1.05 μmol/L), the device has a rmse at 2.93 μg/mL (0.14 μmol/L). And if the cutoff for diagnosing VAD was set to 22 μg/mL (1.05 μmol/L), the device yields sensitivity at 83% and specificity at 91% for VAD diagnosis. (*C*) ROC curve for RBP, using cutoff at 14.7 μg/mL (0.70 μmol/L).

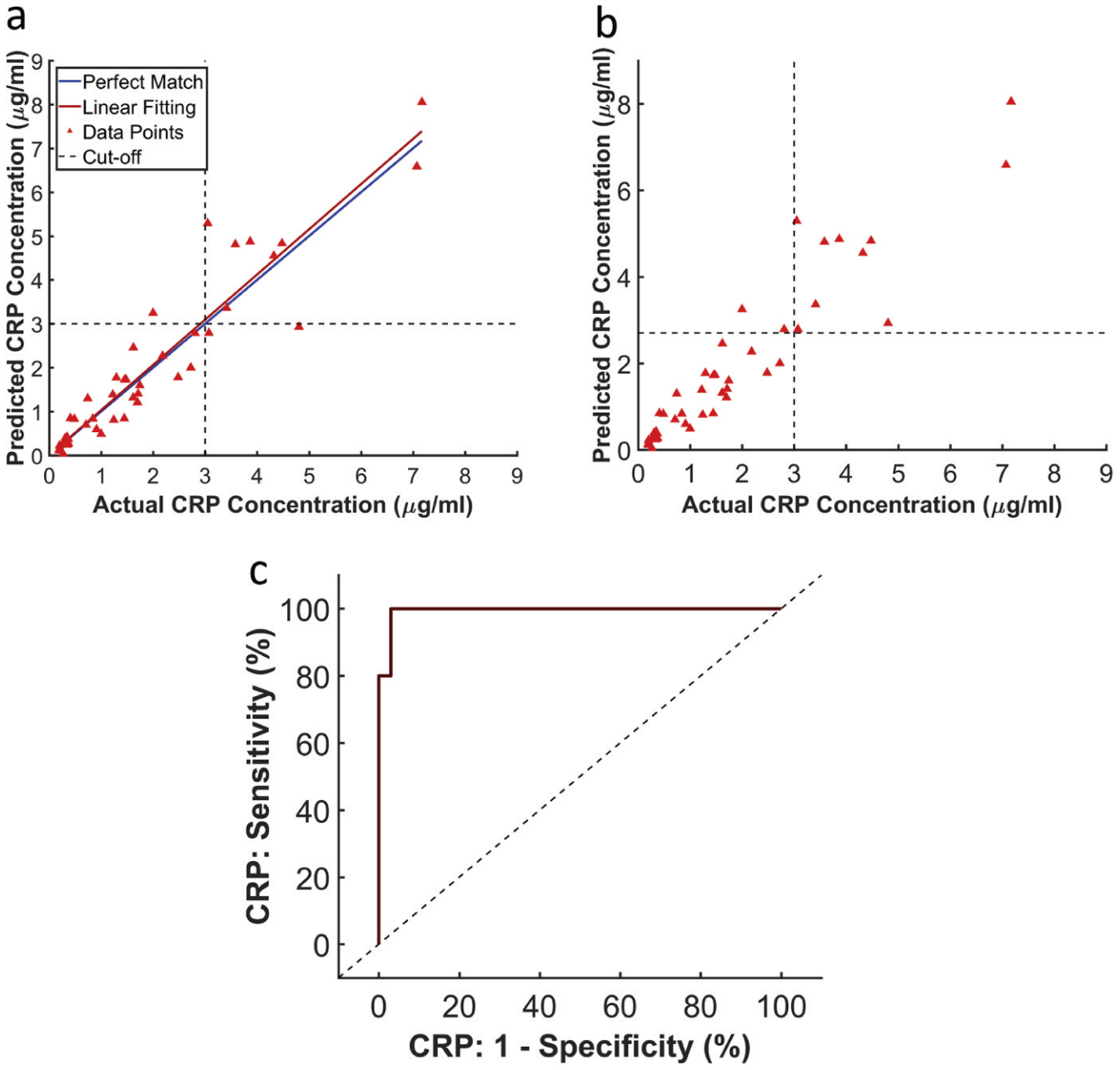

Fig. S8. (*A*) Comparison between actual and predicted concentration for CRP. Red line indicates RC = 1.03. Dashed line indicates cutoff at 3 μg/mL. (*B*) Comparison between predicted and actual concentration for CRP and cutoff at 2.8 μg/mL, when the system yields sensitivity of 100% and specificity of 94%. (*C*) ROC curve for CRP.

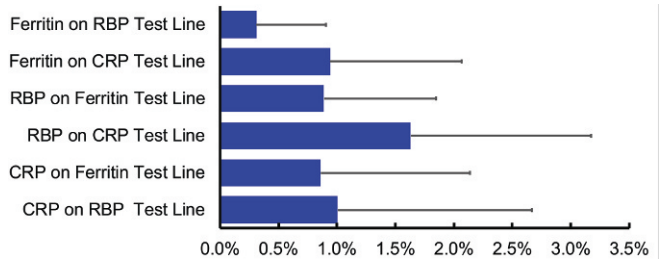

Fig. S9. Level of cross-binding as a percentage of primary binding. Twelve human samples were tested for each type of cross-binding, and the error bars show SD of each data point.

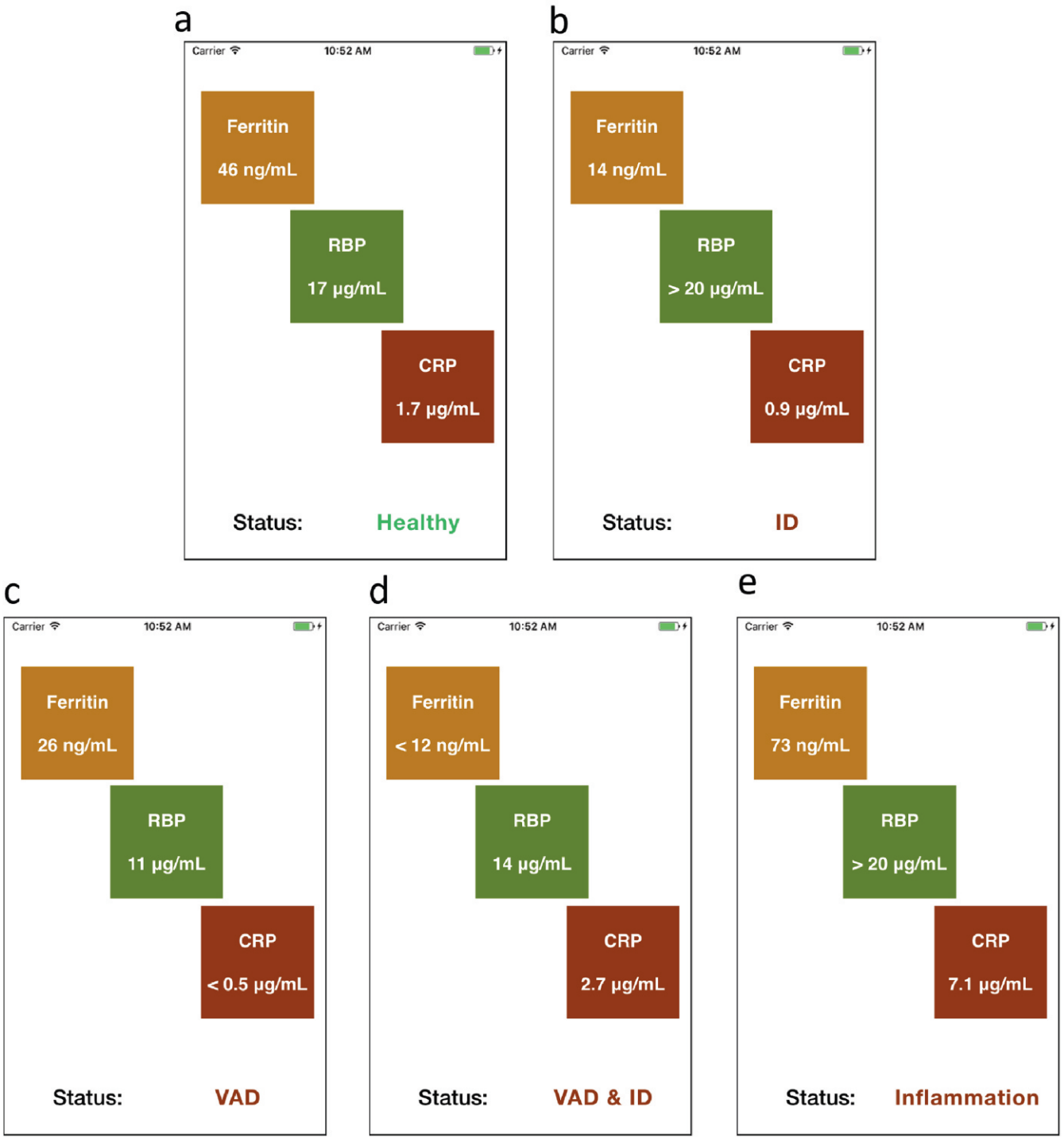

Fig. S10. Screenshots showing quantitative data for other circumstances. (*A*) Sample screenshots for sample diagnosed as healthy with concentrations of all three biomarkers falling in the quantitative range. (*B*) Screenshots for sample diagnosed as ID with RBP concentration larger than 20 μg/mL (0.95 μmol/L). (*C*) Screenshots for sample diagnosed as VAD with CRP concentration less than 0.5 μg/mL (20 nmol/L). (*D*) Screenshots for sample diagnosed as VAD and ID with ferritin concentration less than 12 ng/mL (26 pmol/L). (*E*) Screenshots for sample diagnosed as inflamed with RBP concentration larger than 20 μg/mL (0.95 μmol/L).

Table S1. Values of parameters used to predict biomarker concentration according to brightness value

Parameter	Coefficient	SE	95% CI
Ferritin			
d	26.6202	19.9986	−13.8309 to 67.0712
a	0.8683	0.4386	−0.01889 to 1.7556
c	222.0232	194.7995	−171.9960 to 616.0423
b	1.5278	0.4705	0.5761 to 2.4794
RBP			
d	76.8916	7.3935	61.9369 to 91.8463
a	18.7651	0.9014	16.9419 to 20.5882
c	11.5535	0.8614	9.8111 to 13.2958
b	−5.2785	1.1826	−7.6704 to −2.8865
CRP			
d	28.7426	9.9007	8.7167 to 48.7686
a	3.6945	2.032	−0.4156 to 7.8046
c	3.1046	2.1228	−1.1891 to 7.3983
b	1.0411	0.4826	0.06492 to 2.0173

Regression equation: $Y = d + (a - d)/(1 + (X/c)^b)$. Sample size for ferritin, RBP, and CRP was 43. Residual SD for ferritin = 0.9451; RBP = 4.2275; and CRP = 1.6783. R^2 for ferritin = 0.94; RBP = 0.92; and CRP = 0.90.