Supplemental Information

Measuring Markers of Liver Function Using Micro-Patterned Paper and Blood Obtained From a Finger Prick

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EXPERIMENTAL

Fabricating the Devices

Patterned paper: A commercial printer (Xerox Phaser 8560N color printer) printed hydrophobic wax-based ink on the surface of chromatography paper (Whatman Chromatography 1).[1] One sheet of paper contained 150 individual devices. Each device measured 17 mm^2 and consisted of three white circles (diameter = 3.0 mm) enclosed in a black circle (diameter = 9.0 mm). Heating the paper in an oven (T ~ 150 °C for 90 seconds) melted the wax through the thickness of the paper; the melted wax created a hydrophobic barrier around the three circular hydrophilic zones (diameter ~2 mm). These sheets were cut into individual squares to be assembled into devices (see below).

Lamination sheets: A laser cutter created the top and bottom lamination squares (Fellowes Self-Adhesive Laminating Sheets) (length = 25 mm^2) and holes (diameter = 5.7 mm) in the center of the top lamination square.

Plasma separation membranes (PSM): A laser cutter created disks (diameter = 7.0 mm) from a sheet of Vivid GX (PSM). We assembled the devices manually by centering a disk filter around the hole of the top self-adhesive lamination sheet, and centering a patterned paper device on the bottom self-adhesive lamination sheet, aligning the two parts, and sealing them together. (Figure 2b)

Device Assembly: We assembled each device separately. A plasma filter was centered over the hole of the top lamination sheet, while ensuring that the plasma filter was oriented correctly (the asymmetric nature of the filter required that whole blood be applied to the dull side of the filter). A single patterned paper square was centered on the bottom lamination sheet. The plasma filter

was aligned over the reaction zones, and then the top and bottom lamination sheets were pressed together.

Performing Liver Function Tests

Preparing the devices for assays:

Aspartate aminotransferase (AST). We measured the levels of aspartate aminotransferase in plasma using a modified procedure.[2] Spotting reagents in the following order prepared the reaction zones: i) 0.7 μL of a solution of 10% trehalose in water; ii) 0.7 μL of a substrate solution containing cysteine sulfinic acid (CSA) (306 mg), α-ketoglutarate (34 mg), ethylene diaminetetraacetic acid (EDTA) (1.6 mg) in TRIS buffer (400 mM) (1 mL); iii) 0.7 μL of a reagent solution containing 1% polyvinyl alcohol (250 mg), 0.4 % methyl green (100 mg), 0.2% rhodamine B (50 mg), zinc chloride (2.75 mg), triton X-100 (1 drop) in water (25.0 mL); iv) 0.7 μL of a reagent solution containing 1% polyvinyl alcohol (250 mg), 0.1 % methyl green (25 mg), 0.1% rhodamine B (25 mg), zinc chloride (2.75 mg), triton X-100 (1 drop) in water (25.0 mL). Ten minutes of air-drying at 25 °C followed each addition.

Alkaline phosphatase (ALP). We measured the levels of alkaline phosphatase in plasma using a modified procedure.[3] Spotting reagents in the following order prepared the reaction zones: i) 0.7 μL of a reagent solution containing 0.15% of nitro blue tetrazolium (NBT) and 0.1% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 5% DMSO in water (two times); ii) 0.7 μL of 500 mM Tris buffer (pH 9.5). Ten minutes of air-drying at 25 °C followed each addition.

Total levels of proteins (Protein). We measured the total levels of proteins in plasma using a modification of a known procedure.[4,5] Spotting reagents in the following order prepared the reaction zones: i) 0.7 μL of a 250-mM citrate buffer solution (pH 1.8) containing Triton X-100 in the test area (two times); ii) 0.7 μL of a 6.0-mM tetrabromophenol blue (TBPB) solution in 4%

ethanol in water, iii) 0.7 μL of a 250-mM citrate buffer solution (pH 1.8) containing Triton X-100 (three times). Ten minutes of air-drying at 25 °C followed each addition.

Procedure for running the assays:

We standardized the stock solutions of AST and ALP using a UV-visible spectrometer method according to the vendor specifications (Pointe Scientific, Inc.). Devices prepared with all three reaction-zones containing reagents for one assay generated the calibration curves. (Figure 3a) AST; 3b) ALP; 3c) Total Protein) A minimum of three devices with three detection zones each provided nine intensity values for each concentration. Analyte solutions (0 - 500 U/L for AST, 0 – 1000 U/L for ALP, and 0 – 133 g/L of BSA for Protein) in artificial blood plasma were warmed for 2 minutes at 37 °C. Spotting 15 μL of the analyte solutions to the filter membrane of an assembled device developed the assays. A desktop scanner (CanoScan LiDE 700F from Canon) digitized the developed assays every five minutes for 45 minutes at 800 dpi resolution. ImageJ analyzed the digital images and provided intensity data for each concentration at each timed interval.

ImageJ analysis:

Scanned images of the devices were saved as jpeg files. A microarray plugin for ImageJ (MicroArray_Profile.jar)[6] analyzed the scanned images. We sized the circles of the grid to match the size of the reaction zone; the size of the circles was kept constant for the analysis of each zone on every device for one particular assay. The absolute size of the circles was dependent on the resolution of the image. The size of the circles used to analyze the devices was 100 for AST, 70 for ALP, and 70 for total protein. Each circle was positioned manually over each reaction zone. Once the circles were in place, ImageJ measured the intensities. The

intensities were measured for total color, red, blue, green, and grayscale. We did not perform any background correction.

Calibration curves. The calibration curves for each assay were generated by fitting the color intensities reported from Image J at each concentration to an exponential curve with the general equation: $y = A*\exp(x/t) + y_0$. The values of the parameters for each assay are listed in Table S1.

Table S1.

	y_0	A	t	r^2
AST	127 ± 4	-63 ± 6	-92 ± 21	0.94
ALP	57 ± 2	101 ± 2	160 ± 11	0.99
Total Protein	33 ± 5	169 ± 6	-25 ± 4	0.99

Procedure for cross-reactivity experiments:

Devices were prepared by spotting reagents for each assay in their respective reaction zones. Samples of artificial blood plasma spiked with either AST (780 U/L), ALP (1200 U/L), or BSA (150 g/L) were prepared; 15 μL of one of these solutions was spotted onto seven assembled devices each; three devices were spotted with ABP as controls. Analysis of each assay at the appropriate time and on the appropriate channel determined if each assay was specific for only one analyte.

Cross Reactivity:

The colorimetric assays we chose for the markers of liver function are specific enough to prevent cross-reactivity with the other analytes tested on the device. We tested this hypothesis by assembling regular test devices (each of the three test zones contained the reagents for the

different assays) and applying ABP spiked with only one analyte to the top of the device (unspiked ABP was used as a control). Sufficiently high concentrations of the analytes in ABP (AST (780 U/L), ALP (1200 U/L), or BSA (150 g/L) elicited a response in the reaction zone containing the corresponding assay. Analysis of all zones at the appropriate time and on the appropriate color channel (as determined from the calibration curves) calculated concentrations of analytes in each spiked sample. Figure S1 summarizes the results from this cross-reactivity experiment. A sample device is shown above the intensity bars for each solution tested. The control devices treated with ABP solution showed no response in any of the assays, i.e., the ALP reaction zone remained colorless, the AST reaction zone remained dark blue, and the Protein reaction zone remained yellow. The devices treated with AST only showed a response in the AST assay; the AST assay turned pink and the average calculated concentration was 117 U/L. Similarly, the ALP solution elicited a response only in the ALP assay (it turned purple, and the average calculated intensity was 82 U/L), and the BSA solution elicited a response only in the Total Protein assay (it turned green, and the average calculated intensity was 150 U/L). The discrepancy between the calibrated stock solutions used to elicit a response in the assays and the values calculated from the measured intensities can likely be attributed to the same sources described for the error bars of the calibration curves. These results indicate that cross-reactivity between assays and analytes did not occur; each assay is *specific* for its intended analyte (AS**S**URED - *Specific*).

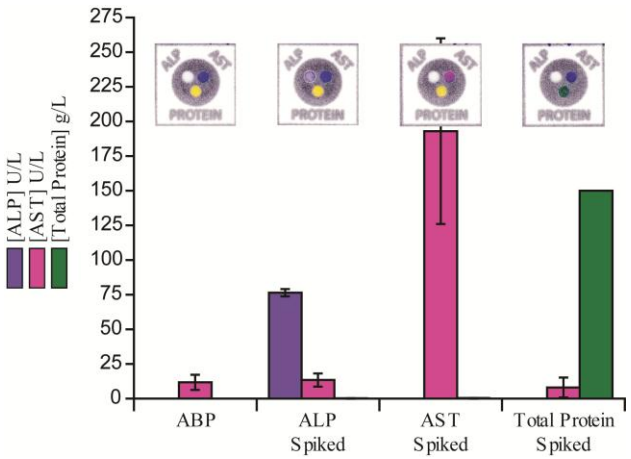

Figure S1. Results from testing the cross-reactivity of the enzymes prepared in artificial blood plasma. Samples were prepared with sufficient quantities of one analyte to elicit a response in each assay. Analysis of the each assay on the devices at the appropriate time and color channel determined the concentration of each analyte. Each series of devices only elicited a response between the analyte and their respective assay, indicating that each assay was specific for its analyte and that no cross-reactivity occurred.

Analysis in whole blood:

Procedure for developing devices using whole blood:

Devices were prepared by spotting reagents for each assay in their respective reaction zones. Blood from a finger prick provided an unspiked sample. A disposable needle pricked the finger; a pipette collected 15 µL of the pooled blood and transferred it onto the filter of the device. We prepared the spiked whole blood samples by adding <1 µL (10-100 nL, i.e. the tip of a 1 uL pipette tip) of AST (stock = 7854 U/ml), <1 µL (10-100 nL, i.e. the tip of a 1 uL pipette tip) of ALP (52600 U/ml), and/or 20 µL of BSA (stock of ~500 g/L) to 200 µL of a sample of blood. We did not accurately prepare the spiked samples of whole blood, nor did we calibrate the actual concentration of the added analytes. The blood theoretically contained ~4000 U/L AST, ~26000 U/L ALP, and/or ~50 g/L total protein.15 µL of the blood samples added to the filters developed the assays. A scanner imaged the bottom side of the devices every five minutes for 45 minutes. Image J analyzed the scanned data on the total color, grayscale, red, green, and blue channels. The concentrations calculated based on the color intensity for the samples analyzed at the specified times for each analyte are summarized in Table S2. The values calculated for AST and ALP in the spiked samples were lower than the theoretical values. The discrepancies could be the result of one or a combination of several factors. i) We generated the calibration curves using a surrogate artificial blood plasma (ABP) and not real blood plasma, which could affect the measured color intensities. The presence of a background color from the red blood cells on the top side of the filter could affect the color intensity of the developed assay. ii) The activities of the enzymes could have been lower than the measured value on the bottle, perhaps due to aging of the stock solution. iii) The variation in the quantity of spotted reagents in the reaction-zones

due to manual fabrication of the devices could potentially alter the rate of color development for a particular assay.

Table S2. Calculated concentration of analytes in whole blood based on intensity values measured at the specific times for each assay.

Sample	**AST**		**ALP**		**Total Protein**	
	Intensity	**[AST] [U/L]**	**Intensity**	**ALP [U/L]**	**Intensity**	**Total Protein [g/L]**
Unspiked	51	<44	171	<15	54	53
Spiked with AST, ALP, Protein	104	92	82	220	48	61
Spiked with AST	102	84	169	<15	65	42
Spiked with ALP	44	<44	100	136	122	16
Spiked with Protein	52	<44	168	<15	32	>150

References

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