

Supplementary Information for:

**Reagent pencils: a new technique for solvent-free deposition of
reagents onto paper-based microfluidic devices**

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FIGURES AND TABLES

	Trial 1 ($\mu\text{g}/\text{mm}^2$)	Trial 2 ($\mu\text{g}/\text{mm}^2$)	Trial 3 ($\mu\text{g}/\text{mm}^2$)	Mean ($\mu\text{g}/\text{mm}^2$)	St. Dev ($\mu\text{g}/\text{mm}^2$)
User 1	3.75	3.75	2.50	3.33	0.72
User 2	3.75	3.13	4.38	3.75	0.63
User 3	3.75	4.38	1.88	3.33	1.30
User 4	3.81	3.50	4.00	3.77	0.25
User 5	3.50	3.75	4.00	3.75	0.25
Overall				3.59	0.66

Table S1. Results from the mass of deposition experiments. User 5 was selected to deposit the reagents for the shelf life experiments.

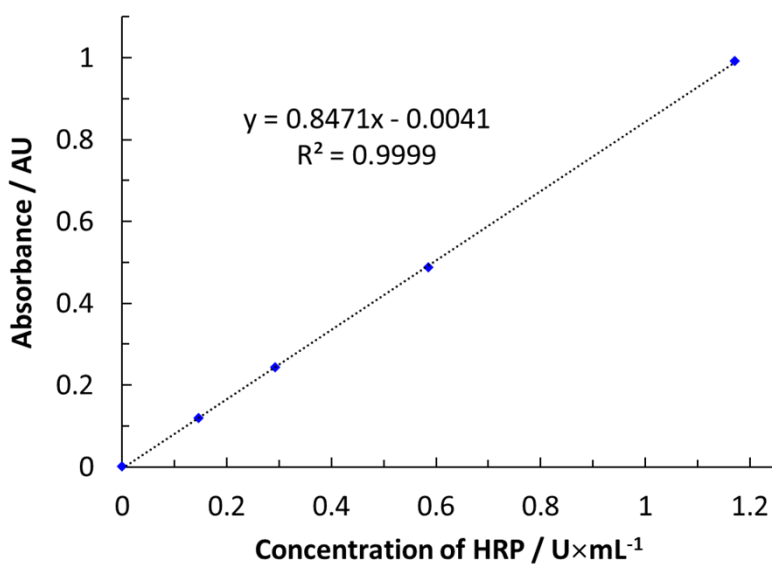


Figure S1. External calibration curve for HRP activity assay.

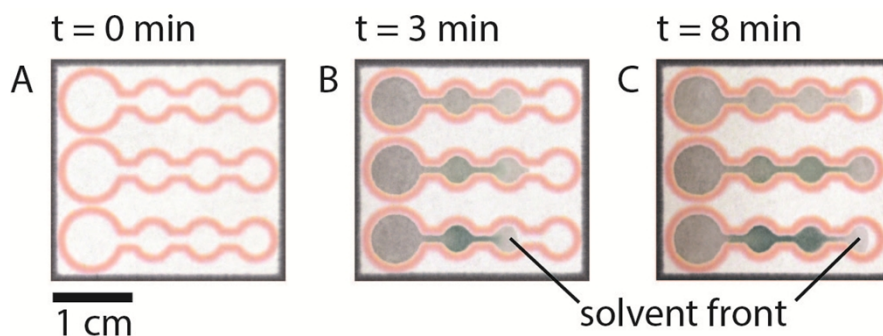


Figure S2. Device for conducting glucose assays shown in Figure 4C 0 min, 3 min, and 8 min after adding the glucose sample solution to the sample inlet. The solvent front with no oxidized ABTS enters the waste zone, while the colored ABTS product fills the test zone.

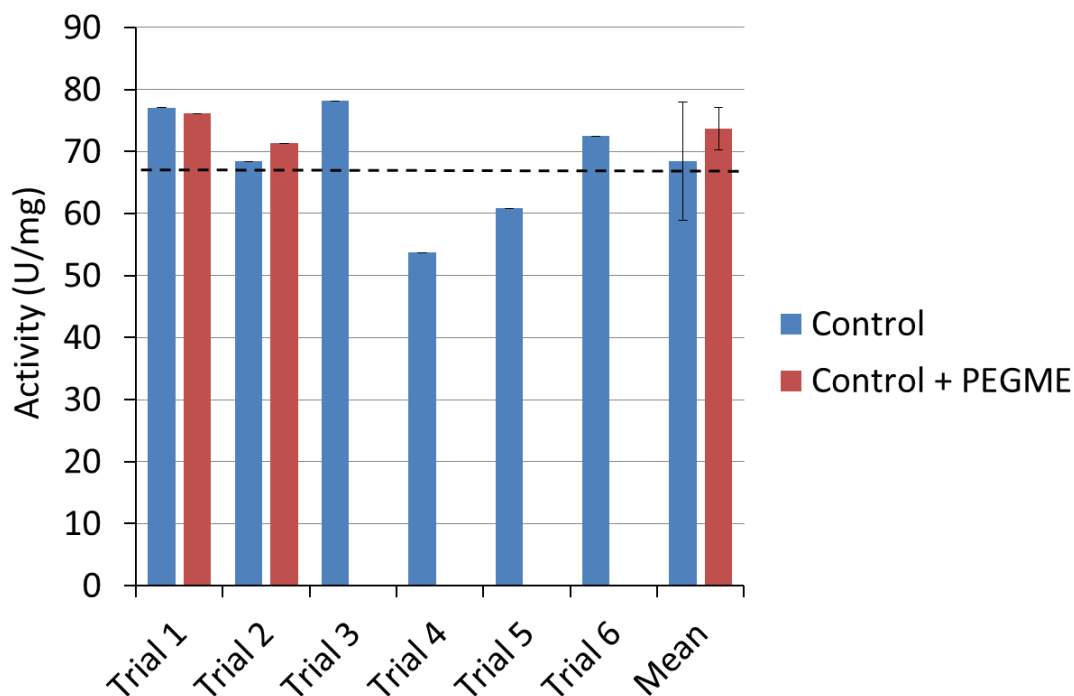


Figure S3. Determined activity of HRP as supplied by the vendor. The reported activity from the vendor was 67 U/mg (black dashed line). For trials 1 and 2, PEGME was also added to the test solution to determine its effect on the activity of HRP in solution. Error bars represent one standard deviation from the mean. The uncertainty in the determined activities can be attributed primarily to the uncertainty in weighing out small masses (2-5 mg) of HRP.

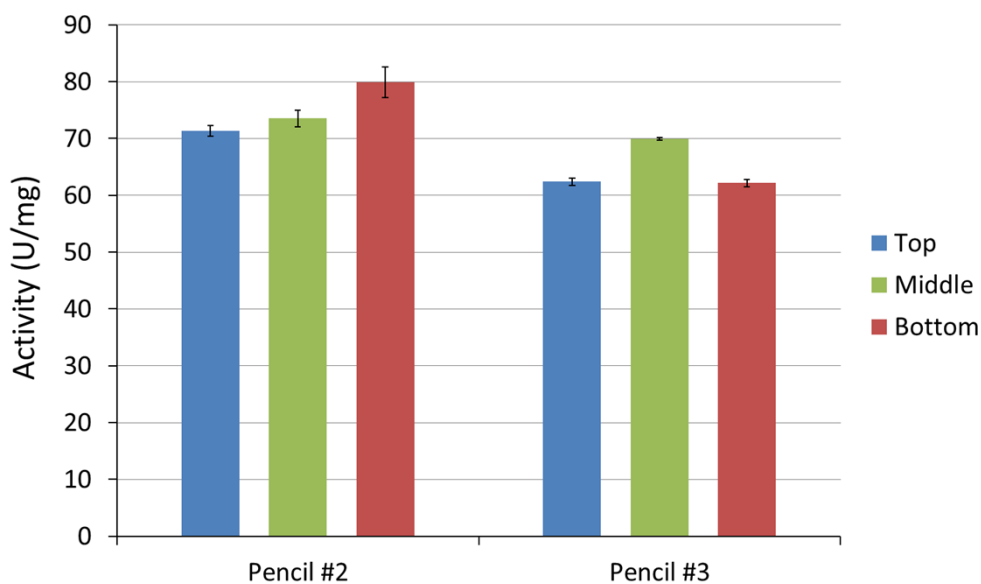


Figure S4. Activity of HRP in different locations (top, middle and bottom) of the pencil core. Pencil #2 and Pencil #3 correspond to the data for Trial 2 and Trial 3 shown in Table 1. Bars represent the mean of three replicates. Error bars represent one standard deviation from the mean.

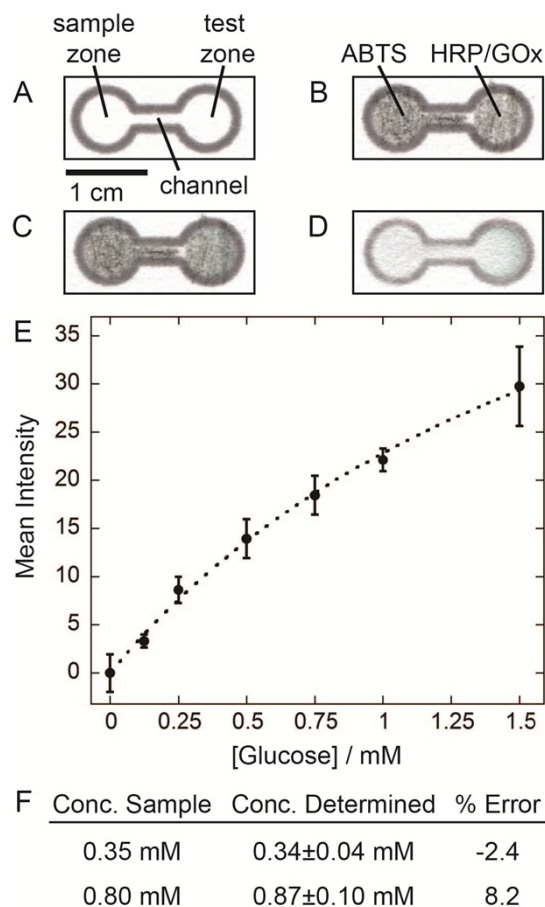


Figure S5. Quantitative glucose assay using MicroPADs prepared with reagent pencils. These tests were conducted 8 months before the glucose tests shown in Figure 4 using the same reagent pencils. A) Device used for the assay. B) Device after depositing the reagents for the test using two different reagent pencils. C) Top-view of the device 30 minutes after adding a sample containing 1-mM glucose to the sample zone. D) Bottom-view of the device shown in C. The results appear as a blue-green color in the test zone. E) External calibration curve prepared by measuring the intensity of the color in the test zone for standard glucose samples. Data points represent the mean of 6 replicates and error bars represent one standard deviation from the mean. F) Results for the determination of the glucose concentration in two calibration samples. Results represent the mean of 6 replicates; the uncertainty is reported as one standard deviation from the mean. Experimental details for these experiments are described below.

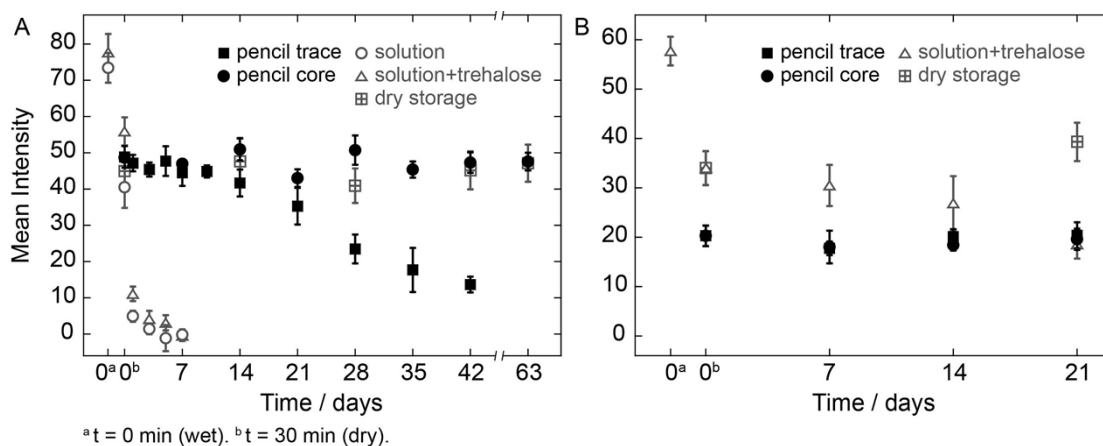


Figure S6. Non-normalized results from stability tests for HRP (A) and ALP (B). The signal for the tests conducted with reagents deposited from solution are higher than the signals for the tests with reagents deposited using reagent pencils because of the large background signal created by the graphite in the test zones. Data points represent the mean of 8 replicates and error bars represent one standard deviation from the mean.

EXPERIMENTAL

Reagents and equipment.

All reagents, materials, and equipment were purchased from commercial sources unless stated otherwise. The following reagents, materials, and equipment were used: poly(ethylene glycol) methyl ether ($M_n \sim 2000$ g/mol, Sigma Aldrich), graphite powder (General's Pure Powdered Graphite), horseradish peroxidase (HRP, 67 U/mg, MP Biomedicals), alkaline phosphatase (ALP, 26 U/mg, MP Biomedicals), glucose oxidase (GOx, 266 U/mg, MP Biomedicals), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Alfa Aesar), 1-StepTM ABTS (a proprietary HRP substrate solution, Thermo Scientific), BCIP[®]/NBT-Purple Liquid Substrate System for Membranes (a proprietary ALP substrate solution, Sigma Aldrich), dextrose (glucose, Sigma Aldrich), 1X phosphate-buffered saline (1XPBS pH 7.4, prepared from a 10X solution, Fisher BioReagents), Erioglaurine disodium salt (blue dye, Alfa Aesar), Allura

red AC (red dye, Alfa Aesar), Tartrazine (yellow dye, Alfa Aesar), chromatography paper (Whatman No. 1, GE Healthcare Life Sciences), porcelain mortar and pestle (Coors Brewing Company), agate mortar and pestle, manual pellet press equipped with a ¼ inch punch and die set (Parr Instrument Company), analytical balance (Sartorius), flatbed scanner (Epson Perfection V300), mechanical pencil holders (Art Alternatives), micropipettes (p1000, p200, p20, p10, p2, Gilson), solid ink printer (Xerox Phaser 8560), convection oven (MTI corporation), non-contact liquid dispenser (Mantis, Formulatrix), centrifuge (Marathon Micro A, Fisher Scientific), and a spectrophotometer (Cary 100 UV-vis, Agilent Technologies).

Fabrication of reagent pencils.

The following reagent pencils were prepared for the work described in this article: 0.50% w/w HRP, 0.50% w/w ALP, 15.0% w/w ABTS, 10.0% w/w enzymes (5.0% w/w GOx and 5.0% w/w HRP), 15.0% w/w Erioglaurine disodium salt (blue dye), 15.6% w/w Allura Red AC, 15.6% w/w Tartrazine (yellow dye), and 15.3% w/w dye mixture (7.5% w/w Erioglaurine disodium salt and 7.5% w/w Tartrazine).

Digital image colorimetry (DIC).

Devices were scanned using the following settings: 48-bit color image type, 300dpi resolution, and reflective document type.^[1,2]

Effect of pencil fabrication on the activity of HRP.

Samples were saved at three points during the fabrication of reagent pencils containing 0.5% w/w HRP: i) before the enzyme was added to the PEGME-graphite mixture as a control sample (6 trials with masses of HRP ranging from 2 to 5 mg), ii) after the enzyme had been mixed with the PEGME-graphite in the agate mortar (3 trials

with masses of HRP-PEGME-graphite mixture ranging from 20 to 60 mg), and iii) after the enzyme had been pressed into the pellet (3 trials with masses of the pellet ranging from 20 to 50 mg). A total mass of 1.0 g of material (0.995 g of PEGME-graphite and 5 mg of HRP) was prepared for each of the three trials. Each sample was dissolved in 1XPBS to achieve a theoretical concentration of HRP of 23 U/mL in microcentrifuge tubes. The mixtures were then centrifuged for 1 min on speed setting #1 (~1000g) to remove the graphite from the samples. The supernatant was then further diluted to a concentration of 0.58 U/mL. The samples from the reagent pencil also contained 1.3 mg/mL PEGME since it was observe to dissolve readily, so an additional control sample was prepared with 0.58-U/mL HRP and 1.3-mg/mL PEGME in 1XPBS to determine the effect of PEGME on the enzyme activity.

Five microliters of the HRP solution were added to a microcentrifuge tube containing 500 μ L of 1-Step ABTS solution and 500 μ L of 1XPBS, and the solution was mixed by hand. After incubating the solution at room temperature for 6 min, 500 μ L of a 0.625M oxalic acid stop solution prepared in DI water were added. The absorbance of the resulting solution was measured at 415 nM using disposable plastic cuvettes with a path length of 0.5 cm. Each sample was tested in triplicate. A calibration curve was also prepared with HRP solutions prepared in 1XPBS in concentrations ranging from 0 U/mL to 1.2 U/mL and used to determine the activity of each sample.

Colorimetric glucose assay (Figure 3)

The results from the external calibration solutions were used to prepare an external calibration curve that was fit in KaleidaGraph with the following rectangular hyperbolic equation:

$$y = \frac{C_1 x}{C_2 + x} \quad (1)$$

The values for C_1 and C_2 for the two calibration curves were found to be:

	C_1	C_2	R^2
Reagent Pencil	117±8	1.2±0.1	0.998
Solution	135±10	1.6±0.2	0.999

These values were used to calculate the concentration of the calibration standards based on the mean intensity of the test zones recorded for these samples.

Colorimetric glucose assay (Figure S3)

A simple paper-based device with a sample inlet, channel and test zone was fabricated, and a reagent pencil containing 10% w/w enzymes (GOx and HRP, 5.0% w/w and 5.0% w/w, respectively) and a reagent pencil containing 15% w/w ABTS were prepared. ABTS was deposited in the sample zone and channel, and the enzymes were deposited in the test zone of the devices. External calibration solutions (12 μ L) containing glucose in concentrations ranging from 0 mM to 1.5 mM prepared in 1XPBS were added to the sample zone. The solutions wicked from the sample zone through the channel to the test zone where a blue-green color developed when glucose was present in the sample. The devices were allowed to dry for 30 minutes and then were scanned and analyzed. Six replicates were performed for each concentration of glucose. The results from the external calibration solutions were used to prepare an external calibration curve that was fit in KaleidaGraph with the following rectangular hyperbolic equation:

$$y = \frac{C_1 x}{C_2 + x} \quad (1)$$

The values for C_1 and C_2 were found to be 69±7 and 2.0±0.3 respectively, and the fit resulted in an R^2 value of 0.997.

Two calibration standard solutions with concentrations of 0.35 and 0.80-mM glucose were also tested following the same procedure. The external calibration curve was used to determine the concentration of these samples in order to evaluate the accuracy of the assay.

Dye deposition and delivery in color wheel device (for table of contents graphic)

A device was fabricated with seven channels extending from a common sample zone into individual test zones. Regent pencils containing blue dye, red dye, yellow dye or no dye were deposited in the channels in specific combinations in order to generate six different colors in the test zones upon the addition of water. Single letter labels adjacent to each test zone denoted the targeted color (R = red, O = orange, Y = yellow, G = green, B = blue, V = violet, Ø = white or no color). After depositing the reagents, DI water (20 µL) was added to the sample zone and allowed to wick across the channels where it dissolved the dyes and transported them into the test zones. The devices were dried under ambient conditions.

REFERENCES

- [1] C. A. Chaplan, H. T. Mitchell, A. W. Martinez, *Anal. Methods* **2014**, *6*, 1296.
- [2] A. W. Martinez, S. T. Phillips, E. Carrilho, S. W. Thomas, H. Sindi, G. M. Whitesides, *Anal. Chem.* **2008**, *80*, 3699–3707.