

## Electronic Supplementary Information

### A Device Architecture for Three-Dimensional, Patterned Paper Immunoassays

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## ***Materials and Methods***

**Materials.** We used the following papers and membranes to fabricate devices: Whatman chromatography paper (Grade 4), Pall absorbent pad (Grade 165), and Pall Immunodyne ABC membrane (0.45  $\mu\text{m}$  pore size). We purchased double-sided adhesives from FLEXcon (Flexmount Select DF021621 clear, removable/permanent adhesive-double faced liner, and Flexmount Select DF051521 clear, permanent adhesive-double faced liner). We purchased monoclonal anti- $\beta$  hCG (Clone 1) conjugated to colloidal gold (“conjugate”) and goat anti- $\alpha$  hCG (ABACG-0500, “capture”) from Arista Biologicals. We purchased bovine serum albumin (BSA) and Tween 20 from Amresco. We purchased two OSOM hCG Urine Control Sets (1: positive hCG concentration of 70 mIU mL<sup>-1</sup>, negative hCG concentration of 0 mIU mL<sup>-1</sup>; 2: positive hCG concentration of 80 mIU mL<sup>-1</sup>, negative hCG concentration of 0 mIU mL<sup>-1</sup>) from Sekisui Diagnostics. We obtained a high, standard concentration of hCG (250 mIU mL<sup>-1</sup>) from Monobind, Inc. We purchased phosphate buffered saline (PBS) from EMD Chemicals Inc. We purchased Hammersten casein from EMD Millipore. We purchased sucrose, sodium borate, sodium chloride, and Proclin 300 from Sigma Aldrich. We purchased SureVue Urine hCG test strips from Fisher Scientific. We purchased urine from normal and pregnant (1<sup>st</sup> trimester) human female donors from Lee Biosolutions.

**Preparation of Solutions.** We prepared borate buffered saline (BBS) using 25 mM sodium borate and 150 mM sodium chloride at pH 8.20. We chose a borate-based buffer because we required a buffer with a slightly alkaline pH and good thermal stability.<sup>1</sup> Borate is also known to have slight bacteriostatic and fungistatic properties.<sup>1</sup> We prepared block solution using 0.1% (v/v) Tween 20, 5% (w/v) sucrose, 1% (w/v) Hammersten casein, and 0.1% Proclin 300 in BBS.

We prepared conjugate buffer using 2% BSA (w/v), 10% (w/v) sucrose, and 0.1% (v/v) Tween 20 in PBS.

**Characterization of Urine.** We used American Metabolic Laboratories (Hollywood, FL) to quantify the concentration of different forms of hCG in urine, which was not originally provided by the vendor. A radiolabeled sandwich immunoassay determined the amount of the intact hCG heterodimer in the samples of urine from normal ( $0.5 \text{ mIU mL}^{-1}$ ) and pregnant ( $30,000 \text{ mIU mL}^{-1}$ ) females. A chemiluminescence immunoassay determined the total amount of  $\beta$ -hCG subunit in the samples of urine from normal ( $<1 \text{ mIU mL}^{-1}$ ) and pregnant ( $134,000 \text{ mIU mL}^{-1}$ ) females.

**Patterning of Wax Barriers onto Layers of the Three-Dimensional Devices.** The device comprises six layers (**Figure 1**). The sample, conjugate, incubation, and wash layers are prepared from Whatman Grade 4 chromatography paper, the capture layer is prepared from Pall Immunodyne ABC membrane, and the blot layer is prepared from Pall Grade 165 paper. We designed the hydrophobic barriers that define the hydrophilic channels within each layer using Adobe Illustrator and we printed them using a Xerox ColorQube 8750 printer.<sup>2</sup> We printed layers of Whatman through the automatic feed, but we printed layers of Immunodyne membrane through the manual feed to minimize damage to the membrane. The blot layer was not patterned. After the layers were printed, we taped them to acrylic frames and placed into a  $150 \text{ }^\circ\text{C}$  oven for 30 seconds to melt the wax.

**Patterning of Layers of Adhesive.** We patterned holes into sheets of adhesive using a custom die and die press. We applied patterned adhesive sheets to the back of the active layers, and aligned adhesive sheets to the layers by eye. We backed the sample, conjugate, capture, and wash layers with the permanent adhesive film. We backed the incubation layer with the

removable adhesive film to facilitate peeling of the device (**Figure S1**). We laminated the completed layers with an Apache AL13P laminator.

**Optimization of Assay Conditions.** We performed preliminary experiments to identify the concentrations of the capture and conjugate antibodies that would result in the best performance of the hCG immunoassay. Our goals for these experiments were to maximize the positive signal and reduce any signal arising from non-specific binding at the capture membrane. Meeting these criteria would result in an assay that was analytically sensitive and specific, which are desirable for qualitative assays that are interpreted by eye. We systematically varied the concentration of both the capture hCG antibody (3, 5, and 7  $\mu\text{L}$  at a concentration of  $1 \text{ mg mL}^{-1}$ ) and the hCG antibody that was conjugated to colloidal gold (3, 5, and 7  $\mu\text{L}$  at a concentration of 5 OD<sup>1</sup>). We performed assays using concentrations of hCG at 0 mIU  $\text{mL}^{-1}$  (negative) and 50 mIU  $\text{mL}^{-1}$  (positive). We determined the most effective combination to be 5  $\mu\text{L}$  of each antibody and we used these conditions for all assays described in the manuscript.

**Treatment of Layers.** The sample, wash, and blot layers did not receive chemical pretreatments.

*Capture Layer Treatment.* We diluted our stock solution of goat anti- $\alpha$  hCG ( $8.41 \text{ mg mL}^{-1}$ ) to a working concentration of  $1 \text{ mg mL}^{-1}$  in PBS. We added 5  $\mu\text{L}$  of diluted goat anti- $\alpha$  hCG to the capture layer, and let it incubate at room temperature for two minutes and then at 65 °C for eight minutes. We blocked the capture layer with 2  $\mu\text{L}$  of the block solution and repeated the drying process.

*Lateral Channel Treatment.* We treated the lateral channel layer with block solution to avoid non-specific binding during the mixing of sample and conjugate. We applied block

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<sup>1</sup> The concentrations of colloidal gold conjugates (40 nm particles) are often provided in units of optical density (OD) as measured by absorbance at  $\lambda=540 \text{ nm}$

solution at volumes that were proportional to the channel length. We treated the 0 mm channel with 2  $\mu\text{L}$  of block. As the channel increased in length, an additional 1  $\mu\text{L mm}^{-1}$  of block was added (i.e., 6 mm lateral channel was blocked with a total of 8  $\mu\text{L}$ ). After application of block solution, we dried the layer at room temperature for two minutes and then at 65 °C for five minutes.

*Conjugate Layer Treatment.* We first treated the conjugate layer with 2.5  $\mu\text{L}$  of 10% (w/v) BSA in PBS, and allowed the layer to dry at room temperature for two minutes and then at 65 °C for five minutes. Following the conjugate layer pretreatment with BSA, we applied 5  $\mu\text{L}$  of the colloidal gold conjugated to anti- $\beta$  hCG at a concentration of 5 OD and repeated the drying process. We used conjugate buffer as the diluent to bring the concentration of the colloidal gold from 50 OD to 5 OD.

**Assembly of Three-Dimensional Devices.** We assembled paper-based devices from bottom to top, beginning with the wash layer. Alignment holes (shown in **Figure S2**) guide the placement of each subsequent layer, which facilitates the assembly of multiple, contiguous devices (typically, strips of 4–8 devices). After assembling all active layers, we laminated each strip of devices. Assembly was completed after adding the blot layer to the bottom of the strip and repeating the lamination process. Assays could be performed on intact strips or individual devices without affecting the performance of the assay.

**Preparation of Samples of hCG.** We prepared solutions of hCG in buffer (10–80 mIU  $\text{mL}^{-1}$ ) from the OSOM hCG Urine Control Sets by using the negative solution as the diluent for the positive solution. We treated the 1<sup>st</sup> trimester (pregnant) and normal female urine (not pregnant) with 1  $\mu\text{L mL}^{-1}$  of Proclin 300 upon arrival to inhibit contamination. Normal female urine served as a diluent for the 1<sup>st</sup> trimester urine. Before performing either paper-based

immunoassays or lateral flow assays, we clarified the urine by centrifugation at room temperature for five minutes using a VWR MiniFuge at 6,000 rpm.

**Operation of the Paper-Based Immunoassay.** We began the assay by depositing 20  $\mu\text{L}$  solutions of hCG (or urine) onto the sample layer. Once the solution wicked through the layer completely, we applied two 15  $\mu\text{L}$  aliquots of wash buffer (0.05% Tween 20 in PBS) in succession. The assay completed after the second aliquot of wash buffer wicked completely through the sample layer. We monitored assay duration times with a digital timer.

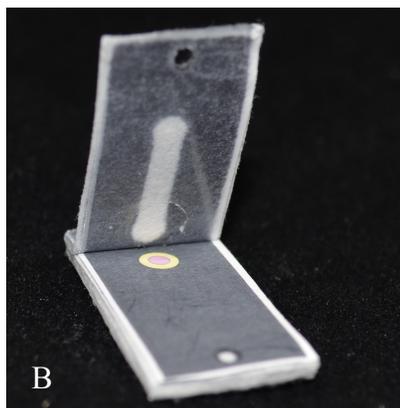
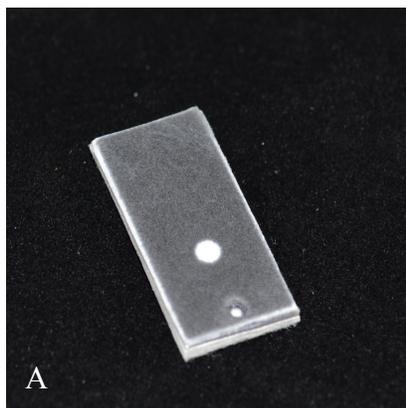
### *Experimental Details.*

**Analysis of Paper-Based Assays.** To determine the results of the paper-based immunoassay, we peeled the top three layers from the capture layer (**Figure S1**), and then scanned the capture layer with an 8-bit EPSON Perfection V500 PHOTO scanner using 48-bit color and a resolution of 800 dpi. We used ImageJ<sup>3</sup> to quantify the signal of the Green channel because it was the most sensitive to the color produced by the colloidal gold. We plotted data in Excel and used Solver to fit the response curve to a one-site Langmuir binding isotherm. We restricted the  $I_{\text{max}}$  variable in Solver to be no greater than 255 because of the limitations of our detection source, an 8-bit scanner. We determined the limit of detection for the paper-based assay as 3-times the standard deviation of the negative sample. (N= 40 negative samples).

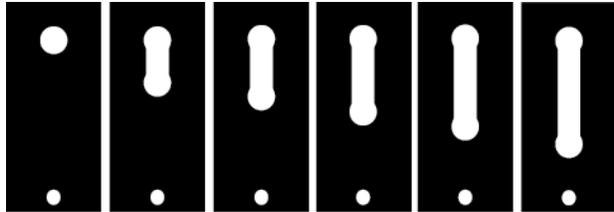
**The Lateral Flow Assay.** We performed lateral flow tests (LFTs) according to the manufacturer's specifications using at least 100  $\mu\text{L}$  of a sample. The sample remained in contact with the device for five seconds, and the results were read within 3–4 minutes. The LFT gave a positive signal at hCG concentrations greater than or equal to 25 mIU  $\text{mL}^{-1}$ . We scanned the LFTs using the same scanner parameters as the paper-based immunoassays, and used the Green

channel to quantify the LFT results. In ImageJ, we analyzed the test line at a specific distance from the control line to ensure consistency between tests for strong positive, weak positive, and negative results. We measured a specific area below the test line and used that as our blank sample for each LFT to normalize the LFT signal (**Figure S3**).

**Figure S1.** Operation of the three-dimensional, patterned paper immunoassay. Images of the paper-based immunoassay fully assembled (A) and after peeling to reveal colorimetric result (B).

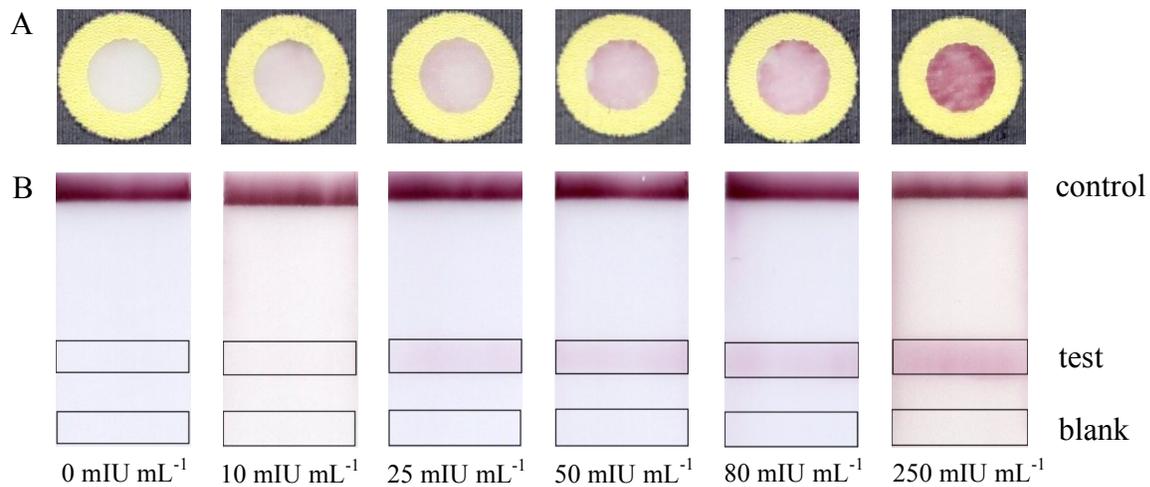


**Figure S2.** Varying length of lateral channels in the incubation layer. The alignment hole, which is included on all layers of the device to guide assembly, is located on the bottom edge on all layers of the device.

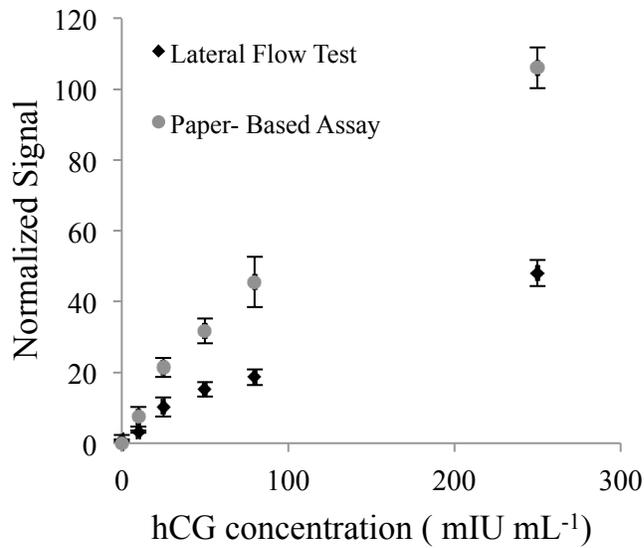


0 mm   2 mm   4 mm   6 mm   8 mm   10 mm

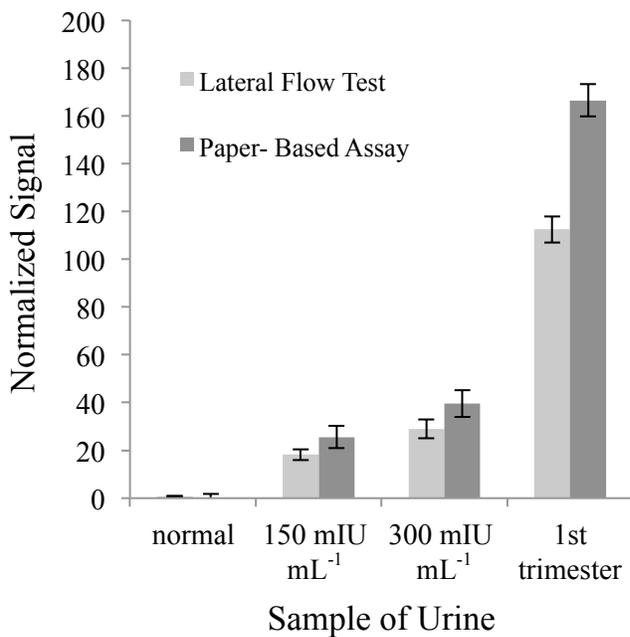
**Figure S3.** Qualitative comparison of lateral flow tests, and three-dimensional, patterned paper immunoassays with standard concentrations of hCG in buffer: 0 mIU mL<sup>-1</sup>, 10 mIU mL<sup>-1</sup>, 25 mIU mL<sup>-1</sup>, 50 mIU mL<sup>-1</sup>, 80 mIU mL<sup>-1</sup>, and 250 mIU mL<sup>-1</sup>. (A) Representative, uncorrected images of three-dimensional, patterned paper immunoassays for hCG in buffer. (B) Representative, uncorrected images of lateral flow tests for hCG in buffer.



**Figure S4.** Comparison of lateral flow tests and paper-based immunoassays for samples of hCG in buffer. Data from paper-based assays represent the mean of eight replicates, and error bars represent the standard deviation of the series. Data from lateral flow tests are the mean of three technical replicates, and error bars are the standard error of the mean.



**Figure S5.** Comparison of lateral flow tests and paper-based immunoassays for hCG with four samples of urine: 1<sup>st</sup> trimester (30,000 mIU mL<sup>-1</sup>), diluted 1<sup>st</sup> trimester (150 mIU mL<sup>-1</sup> and 300 mIU mL<sup>-1</sup>), and normal (0.5 mIU mL<sup>-1</sup>). Data from paper-based assays represent the mean of eight replicates, and error bars represent the standard deviation of the series. Data from lateral flow tests are the mean of three technical replicates, and error bars are the standard error of the mean.



## References

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