Electronic Supplementary Material (ESI) for Analytical Methods.

Electronic Supplementary Information

Comparison of Three Indirect Immunoassay Formats on a Common Paper-Based Microfluidic Device Architecture

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Checkerboard system used to determine immunoassay conditions

We determined the ideal conditions to block non-specific binding of the conjugate reagent to the capture layer by investigating the performance of a number of blocking solutions (e.g., containing skim milk or bovine serum albumin) using devices introduced only to a negative control sample (i.e., buffer). We then determined the optimal conditions for each indirect immunoassay by comparing the responses of immunoassays performed with a number of combinations of concentrations of the capture and conjugate reagents. Our objectives were to (i) maximize the signal we observed from a positive sample, (ii) minimize the non-specific binding of conjugate reagents to the capture layer to reduce background signals, and (iii) limit the amount of reagents needed to perform an assay. For the 1st generation immunoassay, we varied the concentrations of protein G-CG conjugate and unconjugated gp41 capture protein (**Table S1**). For the 2nd generation immunoassay, we varied the concentrations of the gp41-CG conjugate and unconjugated gp41 capture protein (**Table S2**). Finally, for the total IgG capture immunoassay, we varied the concentrations of the gp41-CG conjugate and unconjugated protein G capture protein (**Table S3**). The results given in each table represent the normalized signal of the assay, which is the difference of the negative sample (0 μg mL^{-1}) and the positive sample (10 μg mL^{-1}). The responses indicating the optimal combination of reagents concentrations parameters are italicized and highlighted in red for each table.

Detailed protocol for the treatment of the layers for each immunoassay format

To perform 1st generation immunoassays, we first treated the conjugate layer with 2.5 μL of BSA (100 mg mL^{-1} in PBS), which was allowed to dry at room temperature for 2 minutes and then at 65 °C for 5 minutes. We then treated the same area with 7 μL of 5 OD_{540} protein G-CG, which was diluted from 50 OD_{540} using a conjugate buffer (200 mg mL^{-1} sucrose, 20 mg mL^{-1}

BSA, and 0.1% (v/v) Tween 20 in PBS). We repeated the drying process of the layer after adding the conjugate solution. We applied 5 μL of gp41 (1 mg mL^{-1} in PBS) to the capture layer and allowed it to dry at room temperature for 2 minutes and then at 65 °C for 8 minutes. The extended time was needed to dry the Immunodyne completely. Following the drying of the antigen on the capture layer, we deposited 2 μL of blocking buffer (5 mg mL^{-1} skim milk and 1% Tween 20 in PBS) and then repeated the drying process. While the reagents changed between immunoassay formats, we kept the drying procedures for the treatment steps constant. To perform 2nd generation immunoassays, we added 7 μL of 5 OD_{540} gp41-CG (diluted from 10 OD_{540} with conjugate buffer) to the conjugate layer after first treating the paper with 2.5 μL 10% BSA. We added 5 μL of gp41 (2 mg mL^{-1}in PBS) to the capture layer and then added 2 μL of blocking buffer. To perform total IgG capture immunoassays, we treated the conjugate layer with 7 μL of 5 OD_{540} gp41-CG following treatment with BSA. We applied 5 μL of unconjugated protein G (1 mg mL^{-1}in PBS) to the capture layer and then added 2 μL of blocking buffer.

We treated the incubation and scrub layers with blocking buffer (10 μL and 2 μL, respectively) for all immunoassays. We did not treat the sample and wash layers.

Protocol for performing indirect paper-based immunoassays

We added a sample volume of 20 μL to initiate the immunoassay. After the sample wicked into the device, we sequentially added two 15-μL aliquots of wash buffer (0.05% (v/v) Tween 20 in PBS). We considered the assay to be completed once the second aliquot of wash buffer wicked fully into the device. We then peeled the devices to expose the capture layer, which facilitated the qualitative (by eye) and quantitative (by scanning) analysis of the assay. Commercial samples of human serum were turbid upon thawing. Therefore, to permit the flow of the sample in our device, we clarified them by centrifugation at 10,000 g for the least amount of

time needed while keeping variations in performance by centrifugation to a minimum. Normal human serum required 15 minutes of centrifugation. However, HIV-positive human serum required a longer period of time (20 minutes) to clarify. We added the clarified sera undiluted to paper-based devices and performed the washing and peeling steps as described above. We performed all experiments using HIV-positive serum in a BSL-2+ facility.

Fig. S1. Detailed dimensions of the layers used to fabricate three-dimensional paper-based microfluidic devices for indirect immunoassays.

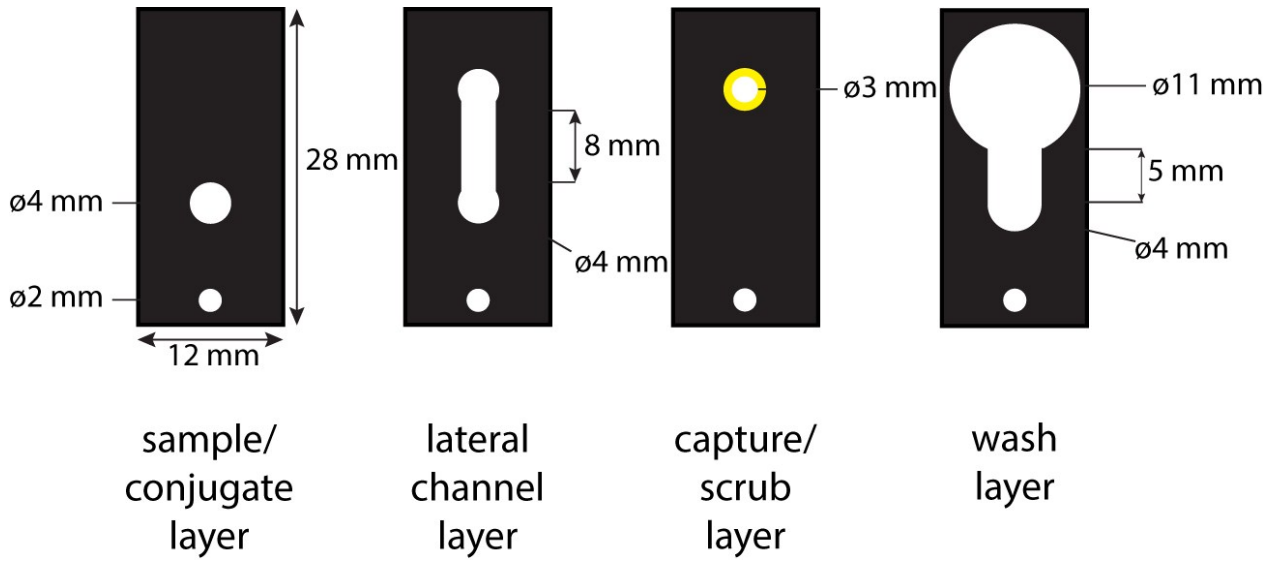

Fig. S2. Representative images of the scrub layer and capture layer for each indirect immunoassay format. The capture layer images are results from the indirect immunoassays for the highest positive sample in buffer (25 μg mL^{-1} HIV gp41 antibody). The scrub layer is not treated with any proteins associated with the immunocomplex, thus the color observed on the layer above the capture layer is considered to be non-specific absorption of conjugate reagents.

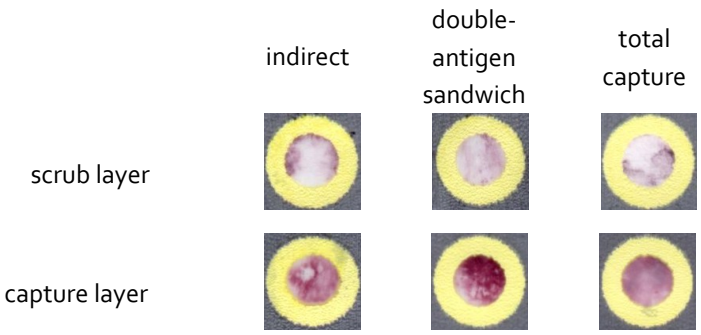

Table S1. Results of the assays performed using the checkerboard system to determine the optimal conditions for the indirect immunoassay.

		Conjugate volume at 5 OD_{540}		
		3 µL	**5 µL**	**7 µL**
Amount of capture protein	**5 µg**	4	26	***50***
	10 µg	11	37	28

Table S2. Results of the assays performed using the checkerboard system to determine the optimal conditions for the double-antigen sandwich immunoassay.

		Conjugate volume at 5 OD_{540}		
		3 µL	**5 µL**	**7 µL**
Amount of capture protein	**5 µg**	76	69	108
	10 µg	82	116	***120***

Table S3. Results of the assays performed using the checkerboard system to determine the optimal conditions for the total IgG capture indirect immunoassay.

		Conjugate volume at 5 OD_{540}		
		3 µL	**5 µL**	**7 µL**
Amount of capture protein	**5 µg**	27	33	***67***
	7 µg	37	59	39

Table S4. Hill equation fitting parameters (I_{max}, K, and n) and the limit of detection (LOD) values for each indirect immunoassay format.

	I_{max}	K	n	LOD (μg mL^{-1})
indirect	152.9	16.6	1.7	4.7
double-antigen sandwich	140.7	3.0	3.0	1.2
total IgG capture	113.5	10.0	1.0	1.8