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Supporting Information

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Paper-Based ELISA**

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Developing the Procedure for an Indirect P-ELISA

For these initial experiments, we used rabbit IgG as a model antigen. To immobilize rabbit IgG on patterned paper, we added 3 μL of a solution of the protein in phosphate buffered saline (PBS) to the test zone, and allowed it to dry for 10 minutes under ambient conditions. We then blocked each test zone by adding 3 μL of a blocking buffer (0.05% (v/v) Tween-20 and 1% (w/v) bovine serum albumin (BSA) in PBS), and allowing the zone to dry for 10 minutes under ambient conditions. A 3 μL solution containing the alkaline phosphatase (ALP)-conjugated secondary antibody in an incubation buffer (0.05% (v/v) Tween-20 in PBS) was added to each zone and allowed to incubate for one minute. Each test zone was washed twice by adding 10 μL of PBS to the test zone, and then bringing the bottom of the paper-microzone plate in contact with a piece of blotting paper to absorb the excess wash buffer from the test zone. Finally, 3 μL of a solution of the colorimetric substrate for alkaline phosphatase (2.7-mM 5-bromo-4-chloro-3-indolyl phosphate disodium salt, 1.8-mM nitrotetrazolium blue chloride, 5-mM $MgCl_2$, 100-mM NaCl, 0.05% Tween in 100-mM Tris buffer, pH 9.5)[1] was added to the test zone immediately after the washing step, and the color-producing enzymatic reaction was allowed to proceed for 30 minutes under ambient conditions. The test zone was scanned, and the intensity of the color was measured using ImageJ.[2,3]

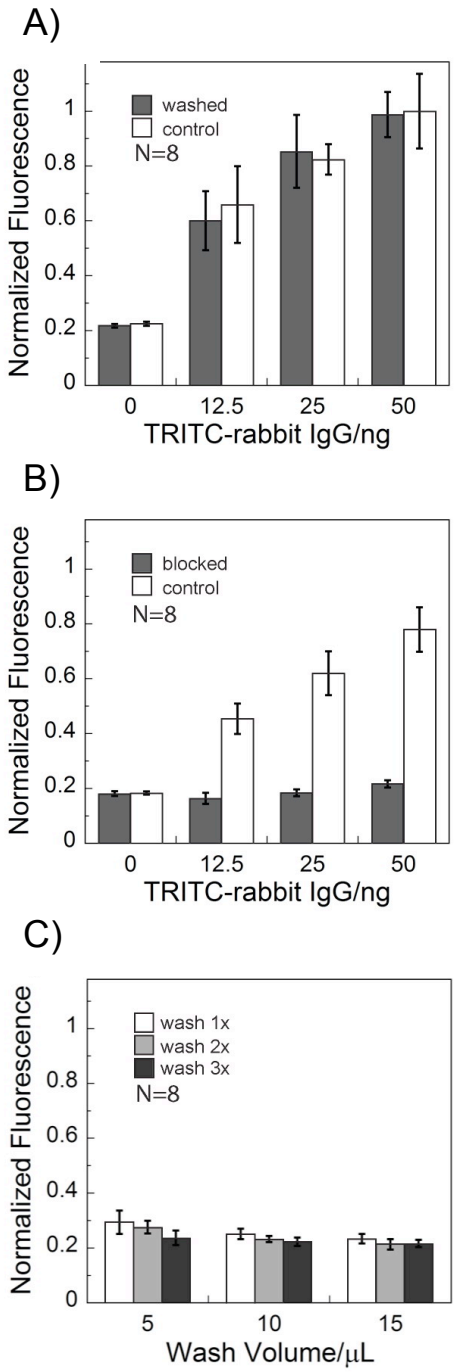
We tested the ability to adsorb antigens and antibodies on paper using solutions of rabbit IgG labeled with tetramethyl rhodamine isothiocyanate (TRITC). TRITC-IgG provided a useful test protein because it could be quantified directly on the paper using a fluorescence scanner without the need for enzymatic catalysis to generate a colorimetric readout. The fluorescence signals of test zones that had been spotted with solutions of

TRITC-IgG, dried, and then washed with PBS were indistinguishable from the fluorescence signals of test zones that had been spotted with TRITC-IgG and not washed. The similarity of these two results indicated that the antibodies were adsorbed to the paper (Figure S1A), and did not elute with the washing buffer.

In order to test our ability to block non-specific binding to the test zones, we spotted 1 μL of a 1% BSA solution on bare paper test zones and allowed the solution to dry for 10 minutes (Figure S1B). We then added solutions of TRITC-IgG to blocked and unblocked test zones. The blocked test zones had fluorescent signals that were equivalent to the background signal; this observation indicated that the blocking step prevented non-specific binding of antibodies to paper. We then tested nine different combinations of washing steps to remove unbound proteins from the paper microzones (Figure S1C). A solution of TRITC-IgG (50 ng/μL) was incubated on blocked test zones for one minute and then washed. Though several different combinations of washes provided complete removal of the labeled antibody, we chose to wash the test zones with two 10 μL volumes of PBS, as this procedure effectively removed traces of non-specifically bound protein while minimizing the time these steps contributed to the assay.

Figure S1. A) Comparison of the fluorescence signal from test zones with immobilized TRITC-labeled rabbit IgG that were either washed or not washed (control). B) Comparison of the fluorescence intensity from bare test zones that were either blocked or not blocked (control), then incubated with solutions of TRITC-IgG for one minute and washed. C) Comparison of the fluorescence intensity from test zones that were blocked, incubated with 50 ng/μL TRITC-IgG for one minute and then washed with PBS using different protocols. The height of the bars for all results shown in (A), (B) and (C) represent the average normalized fluorescence intensity of eight independent measurements, and the error bars represent one standard deviation from the average. All results shown in (A), (B) and (C) were normalized to the average fluorescence intensity of 50 ng of TRITC-IgG immobilized in the paper microzones.

Figure S1.


A)
Normalized Fluorescence
washed
control
N=8
0
0.2
0.4
0.6
0.8
1
0
12.5
25
50
TRITC-rabbit IgG/ng
B)
Normalized Fluorescence
blocked
control
N=8
0
0.2
0.4
0.6
0.8
1
0
12.5
25
50
TRITC-rabbit IgG/ng
C)
Normalized Fluorescence
wash 1x
wash 2x
wash 3x
N=8
0
0.2
0.4
0.6
0.8
1
5
10
15
Wash Volume/μL

Experimental Details

Reagents and Materials. All antibodies and reagents used in this study were purchased from Sigma Aldrich. The HIV-1 envelope antigen gp41 was purchased from ViroGen (Cambridge, MA) and the serum samples were purchased from Golden West Biologicals (Temecula, CA).

Fabrication of a Paper 96-Microzone Plate. We fabricated the 96-microzone paper plate using photolithography[4]. Paper was impregnated with SU-8 photoresist (MicroChem), baked on a hot plate (10 minutes, 110 °C), cooled to room temperature, and exposed to UV light (13 seconds, IntelliRay 600, UVitron International, Inc.) through a transparency mask. The paper was then baked a second time (5 minutes, 110 °C), cooled, and the patterns were developed in an acetone bath (1 minute) followed by a rinse in acetone and a rinse in 70% isopropyl alcohol. The paper was blotted between two paper towels, rinsed a second time with 70% isopropyl alcohol, blotted, and allowed to dry under ambient conditions for at least 1 hour before use.

Immobilizing Proteins on Paper. Solutions of 50 ng/μL, 25 ng/μL, 12.5 ng/μL, and 0 ng/μL TRITC-IgG in PBS (3 μL) were spotted in paper test zones and allowed to dry under ambient conditions. Half of the test zones were washed with PBS (2 × 10 μL) by adding PBS to the test zone and then bringing the bottom of the test zone in contact with a piece of blotting paper. Once dry, the test zones were scanned using a fluorescent scanner (Typhoon Trio, General Electric Co.; set to 526-nm excitation, 532-nm emission). The fluorescence intensity of each test zone was quantified using ImageQuant.

Blocking the Paper. Test zones were blocked with a blocking buffer (1 μL of 0.05% (v/v) Tween-20 and 1% (w/v) BSA in PBS) and allowed to dry for 10 minutes.

Solutions of 50 ng/μL, 25 ng/μL, 12.5 ng/μL, and 0 ng/μL TRITC-IgG in PBS (3 μL) were spotted in test zones that were either blocked or not blocked. After incubating for one minute, the test zones were washed with PBS (2 × 10 μL) as described above. Once dry, the test zones were scanned and the fluorescence intensity of each zone was measured.

Removing Unbound Proteins from Paper by Washing. Solutions of 50 ng/μL and 0 ng/μL TRITC-IgG in PBS (3 μL) were spotted in blocked test zones, incubated for one minute, and then washed using nine different washing protocols. The washing protocols tested the number of washes (from 1 to 3) and the volume of PBS used for each wash (5, 10 and 15 μL). Once dry, the test zones were scanned and the fluorescence intensity of each zone was measured.

Indirect P-ELISA for Rabbit IgG Detection. Rabbit IgG (3 μL) in ten fold dilutions (67 mM to 670 nM, corresponding to 67 pmoles to 670 amoles of IgG per zone spotted on the paper) was immobilized in test zones; PBS was used as a negative control. The test zones were blocked, and a solution of ALP-conjugated anti-rabbit IgG produced in goat (3 μL of a 1:1,000 dilution the of stock antibody solution in 0.05% (v/v) Tween-20 in PBS) was added to the test zones and allowed to incubate for one minute. The test zones were then washed with PBS (2×10 μL), and the ALP substrate solution (3 μL of 2.68 mM BCIP, 1.8 mM NBT, 5 mM $MgCl_2$, 100 mM NaCl, 0.05% (v/v) Tween-20 in 100mM Tris buffer, pH 9.5) was added to each well. After 30 minutes, the test zones were scanned (Perfection 1640SU scanner, EPSON Corp., set to color photo scanning, 600 dpi resolution), and the image was analyzed in grayscale using ImageJ.

P-ELISA for HIV-1 Envelope Antigens Detection. Purified HIV-1 envelope antigen (gp-41, 1 mg/mL) was diluted 1/10 and 1/100 in PBS and then immobilized in test zones. The test zones were blocked using 1% (w/v) BSA. The serum from HIV-1 infected patients and healthy control patients was added to the test zones, incubated and washed. A solution of ALP-conjugated anti-human IgG produced in goat (3 μL of a 1:1,000 dilution of the stock antibody solution in 0.05% Tween-20 in PBS) was then incubated in the test zones and washed. The procedure is the same as that we developed for detecting rabbit IgG. Five microliters of the ALP substrate solution was added to each well; after 30 minutes, the results were scanned and analyzed in ImageJ.

References

(1) J. McGadey, *Histochemie* **1970**, *23*, 180-184.

(2) M. D. Abramoff, P. J. Magelhals, S. J. Ram, *Biophotonics Int.* **2004**, *11*, 36-42.

(3) public software from National Institutes of Health; http://rsbweb.nih.gov/ij/

(4) A. W. Martinez, S. T. Phillips, M. J. Butte and G. M. Whitesides, *Angew. Chem. Int. Ed.* **2007**, *46*, 1318–1320.