Microfluidic Electrochemical Immunoarray for Ultrasensitive Detection of Two Cancer Biomarker Proteins in Serum

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Supporting Information:

Microfluidic Setup:

Using a PDMS kit from Dow Corning, PDMS base material and curing agent were mixed in 10:1 ratio, vigorously stirred for 5 min, then the air bubbles were removed by degassing the mixture for 30 min under vacuum. The clear solution was poured onto I shaped negative mold, and heated at 85 °C for 2.5 h. After cooling, the I shaped PDMS was pealed off the mold, and placed on a 8 electrode array made of carbon ink printed electrode (dia. 700 microns), between two flat, machined poly(methylmethacrylate) (PMMA) plates to provide a microfluidic channel. The channel is 1.5 mm wide, 2.8 cm long and 63 µL volume. The top PMMA plate was equipped with female ports (4 mm diameter) for screwing in standard plastic fittings (1.5 mm i.d., Upchurch) to hold connecting 0.2 mm i.d. tubing (PEEK) for an inlet and an outlet. The top PMMA substrate is also equipped with two holes (0.6 mm for Ag/AgCl and 0.2 mm for Pt wire) near an

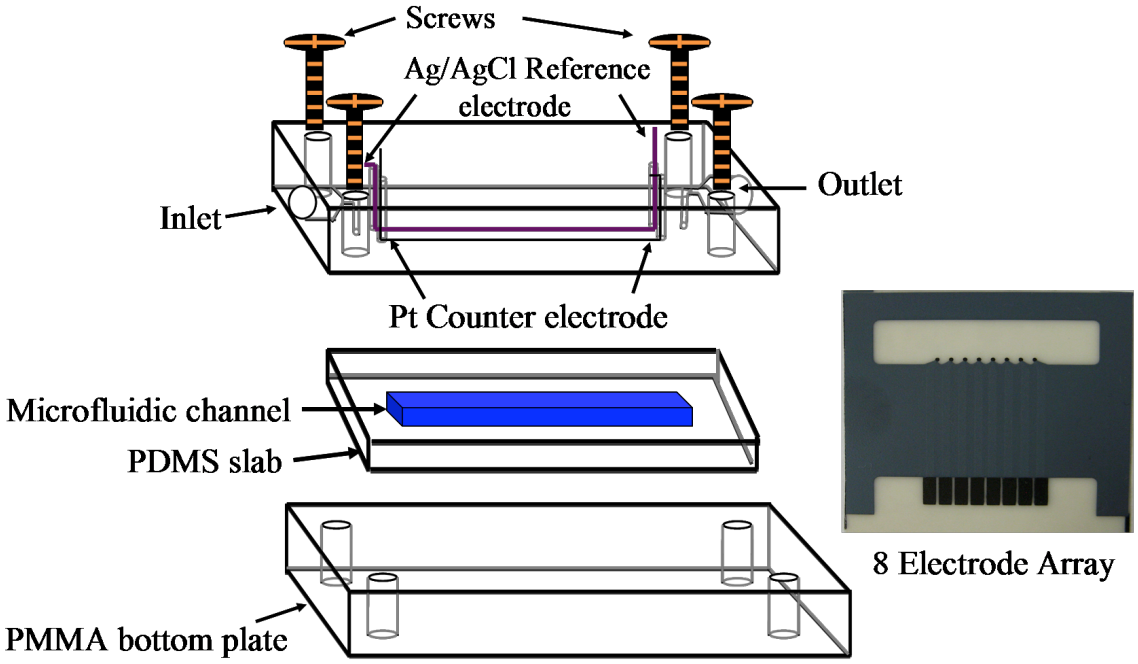

Figure S1. Components of the microfluidic device made of micro-machined poly(methylmethacrylate), PDMS microfluidic channel and screen printed 8 electrode carbon array from Kanichi, Ltd.

inlet and an outlet directly above the microfluidic channel for plugging Ag/AgCl reference and Pt counter electrode wires. Syringe pump (Harvard, 55-3333) was used to inject the samples via an injector valve (Rheodyne, 9725i) which was connected to the inlet via 0.2 mm i.d. tubing.

Full Experimental Sections

Horseradish peroxidase (HRP, MW 44000, 250–330 unit mg^{-1}), L-Gluthathione reduced (GSH, 99%), $HAuCl_4 \cdot 3H_2O$ (99.9%), sodium borohydride (99%) and poly(diallydimethylammonium chloride) (PDDA, MW 20%), bovine serum albumin (BSA) were from Sigma. 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (Sulfo-NHS) from Sigma were dissolved in water (400 mM EDC; 100 mM sulfo-NHS) immediately before use. Hydrogen peroxide (H_2O_2, 30%) was from Fisher. The poly(dimethoxy)silane (PDMS) kit was from Dow Corning. Tosyl-activated magnetic microparticles (MP, Dynabeads®, 1 μm diameter) were from Invitrogen. Tween-20 was from Sigma-Aldrich. Monoclonal (Mouse) primary anti-human prostate specific antigen (PSA) antibody (Ab_1, clone no. CHYH1), tracer secondary anti-PSA antibody (Ab_2, clone no. CHYH2) were obtained from Anogen/Yes Biotech Lab, Ltd. Prostate Specific Antigen (PSA) standard was from Sigma-Aldrich. Monoclonal anti-human Interleukin-6 (IL-6) antibody (clone no. 6708), recombinant human IL-6 (carrier-free) and human IL-6 polyclonal antibody (goat IgG) were from R&D systems. Human serum samples were obtained from Capital Biosciences (Rockville, MD). Immunoreagents were dissolved in pH 7.0 phosphate saline (PBS) (0.01 M in phosphate, 0.14 M NaCl, 2.7 mM KCl) unless otherwise noted. Electrode arrays were from Kanichi Research Ltd. (UK). Protein standards were prepared in diluted calf serum, which has similar properties to human serum but lacks human proteins.

Instrumentation. An eight-electrode CHI 1030 electrochemical workstation connected to the microfluidic array system (Figure 1) was used for amperometry at ambient temperature (22±2 ºC). Amperometry was done at optimal conditions for high sensitivity and low S/N, i.e. -0.2 V vs Ag/AgCl (0.14 M NaCl) reference and solution flow 100 μL min^{-1}. The microfluidic system featured a molded soft PDMS slab with a 1.5 mm wide rectangular channel placed on top of an electrode array featuring 8 carbon screen printed electrodes (dia. 700 microns). This assembly was supported by 2 hard flat poly(methylmethacrylate) (PMMA) plates machined to fit on either side of the PDMS slab, and bolted together tightly to provide a sealed microfluidic channel (Figure S1) 1.5 mm wide, 2.8 cm long, with 63 μL in volume. The top PMMA plate was equipped with female ports (4 mm diameter) for screwing in standard male plastic fittings (1.5 mm i.d., Upchurch) to hold 0.2 mm i.d. PEEK connecting tubing for an inlet and an outlet. The top PMMA substrate is also equipped with two holes, 0.6 mm for Ag/AgCl and 0.2 mm diameter for Pt wire electrode, directly above the microfluidic channel for inserting Ag/AgCl reference and Pt counter electrode wires. A Harvard model 55-3333 syringe pump was connected to the inlet via a Rheodyne Model 9725i injector valve with sample loop via 0.2 mm i.d. tubing.

Glutathione-decorated gold nanoparticles (AuNPs) with diameter 5.0±1.4 nm were prepared as reported previously (Zheng and Huang, 2004). The AuNP immunoarray platform was fabricated by using the layer-by-layer (LbL) alternate electrostatic adsorption on each electrode (Rusling, 2000; Lvov, 2001). A layer of polycation was first adsorbed from aqueous solution by depositing 30 μL drops of 2 mg mL^{-1} polydimethyldiallylammonium (PDDA) onto each array electrode. Then, after washing with water a layer of negatively charged 5 nm GSH-AuNPs was adsorbed from 30 μL drops of 2 mg mL^{-1} aqueous dispersion, resulting in a coating of the array electrodes with densely packed AuNPs (Mani et al., 2009). The electrochemical surface area after coating with AuNP was $2.53 \pm 0.26 \times 10^{-3}$ cm^2, estimated using the Randles-Sevcik equation from the slope of peak currents of soluble 1 mM ferrocyanide in 0.1 M KCl vs square root of scan rate ($\nu^{1/2}$). The layer of AuNPs on the immunoarray greatly improved electrochemical reversibility toward ferrocyanide and resulted in 102% increase in surface area when compared to bare electrodes (Figure S2).

Array characterization:

The AuNP immunoarray platform was fabricated by using the layer-by-layer (LbL) alternate electrostatic adsorption approach (Rusling, 2000; Lvov, 2001). First, a layer of cationic polydimethyldiallylammonium (PDDA) (2 mg mL^{-1}) was adsorbed from 30 µL drops aqueous solution onto array electrode. Then, after washing with water, a layer of negatively charged 5 nm GSH-AuNPs was adsorbed from 30 µL aqueous dispersion (2 mg mL^{-1}) to coat the array electrodes with densely packed AuNPs (Mani et al., 2009). The electrochemical surface area of the array after coating with AuNP was 2.53±0.26 x 10^{-3} cm^2 which was estimated using Randles-Sevcik equation from the slope of peak currents of soluble 1 mM ferrocyanide in 0.1 M KCl vs square root of scan rate ($\nu^{1/2}$). The presence of AuNPs on the immunoarray resulted in 102% increase in surface area when compared to bare electrodes. Figure S2 shows the cyclic voltammogram of 1mM ferrocyanide with bare and AuNPs modified array.

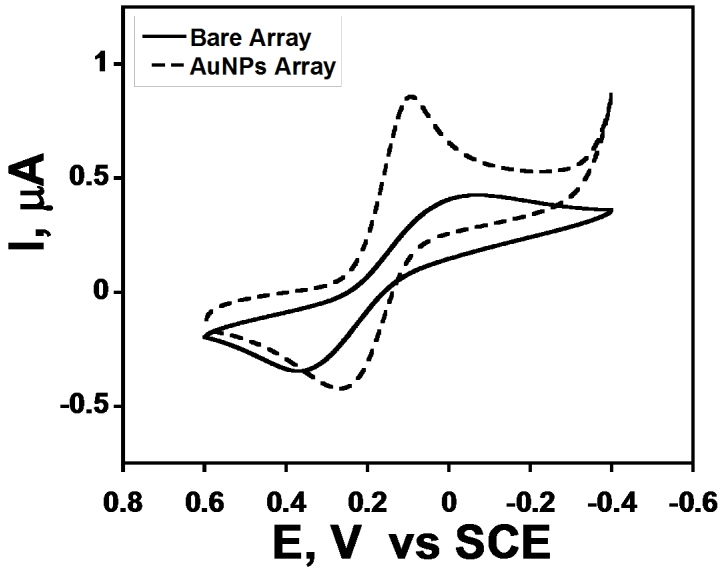

Figure S2. Cyclic voltammogram of 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl at 100 mV s^{-1} with bare array, and AuNPs modified array.

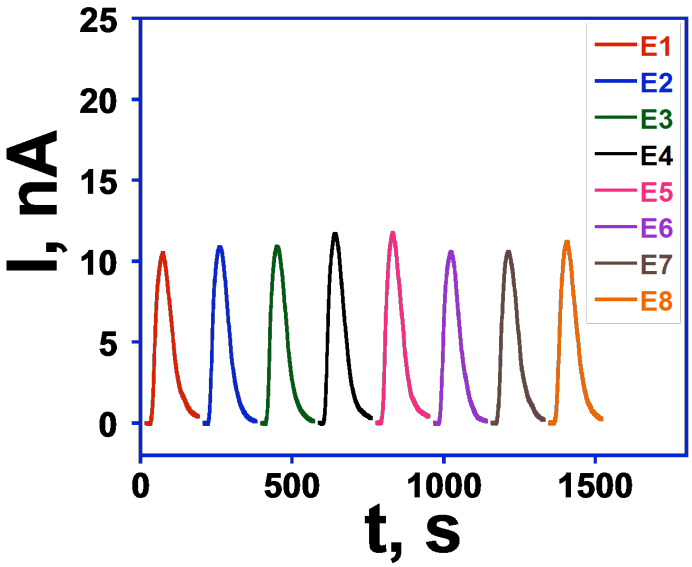

Figure S3. Amperometric response at -0.2 V vs. Ag/AgCl on the 8- AuNP-coated electrodes in the microfluidic array after injecting 100 µL of 100 µM $[Ru(NH_3)_6]Cl_3$ at 100 µL min^{-1}. Average peak current of 10.9 ± 0.4 nA demonstrates minimum cross talk between neighboring electrodes of the array. The electrode-to-electrode standard deviation of <4% is less than that of the voltammetrically determined surface area 2.53±0.26 x 10^{-3} cm^2

Preparation of magnetic particle-Ab_2-HRP conjugates. Secondary antibodies (Ab_2) and HRP labels were conjugated onto 1 μm diameter tosylated superparamagnetic MPs following a protocol supplied by Invitrogen. Briefly, 20 μL of MP (100 mg mL^{-1}) were washed twice with 0.1 M borate buffer (pH 9.5), then 80 μL of Ab_2 (1 mg mL^{-1}) in 625 μL borate buffer containing 1 M ammonium sulfate was added to the MP dispersion to covalently link lysine amino groups of Ab_2 to the tosylated MPs. The reaction was done at 37°C for 24 h with slow tilt rotation in 1.5 mL microcentrifuge tubes. MP-Ab_2 conjugates were separated magnetically, using an Invitrogen DynaMag-spin magnet (cat. No. 123.20D), then mixed with blocking agent 0.5 % BSA in 20 mM phosphate buffer (pH 7.4) and 3 mg mL^{-1} HRP overnight at 37°C. BSA-blocked MP-Ab_2-HRP particles were magnetically separated and washed 4X with 0.1 % BSA in phosphate buffer, then reconstituted in 20 mM phosphate buffer (pH 7.4), and stored at 4°C until use. The MP-Ab_2-HRP bioconjugates were used for 2-3 weeks without noticeable degradation in performance.

Using a BCA protein assay kit (Thermo Scientific, IL, USA), we estimated the number of Ab_2 molecules per MP, by difference, from the amount left in solution, as ~9(±3) x 10^4. The number of horseradish peroxidase labels per magnetic particles was calculated by measuring enzyme activity using 2,2`-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) assay (ABTS) as reactant (Putter, 1983) to be 2.4 x 10^5.

Off-line protein analyte capture. For analyte capture, 20 μL of MP-Ab_2-HRP bioconjugate for each analyte were added to 320 μL of 20 mM phosphate buffer, pH 7.4. For standard calibrations, 20 μL of pure PSA and IL6 in 5000-fold serially diluted calf serum were added to the mixture and incubated with the MP conjugates at 37°C for 30 min using slow tilt rotator. These dilutions were made to correspond to the dilution necessary to bring patient serum PSA and IL-6 levels down into the linear range of the assay. Patient serum was also serially diluted 5000 times for analysis, and 40 μL of the diluted samples were incubated with the mixtures of the MP-Ab_2-HRP, one with Ab_2 = anti-PSA and the other with Ab_2 = anti-IL-6. The resulting MP-Ab_2-HRP-antigen conjugates were magnetically separated and washed 4x (1 min each) with 400 μL of 0.5% BSA in 20 mM phosphate buffer (pH 7.4), made up to 125 μL, and used immediately for immunoassay.

Fabrication of the Microfluidic Immunoarray: The schematic representation of the microfluidic immunoarray is illustrated in Figure S4. We attached the capture antibodies (Ab_1) i.e. anti-PSA and anti-IL6 antibodies each on 4 spots of the array by amidization onto the carboxylated AuNPs of the immunoarray by incubating the AuNPs modified array overnight with 1.2 nmol mL^{-1} anti-PSA antibodies and 2.4 nmol mL^{-1} anti-IL6 antibodies at 4 ºC. Capture antibody coated arrays were washed with PBS-Tween 20 and PBS buffer and inserted into the microfluidic device for further assay development. Inhibition of nonspecific binding (NSB) of labeled magnetic particles (MP) was crucial to achieve ultra high sensitivity and low detection limit. Offline capture of analyte was employed to reduce the NSB of potential interferants in the serum and achieve good sensitivity. Analytes (PSA and IL6) were captured to the magnetic particle conjugates offline (see preparation of the MP-Ab_2-HRP-Ag conjugates) and 2% BSA blocking solution (0.02 M pH 7.0 PBS + 0.05% Tween 20) was used to block the unoccupied primary antibody coated surface. 2% BSA blocking

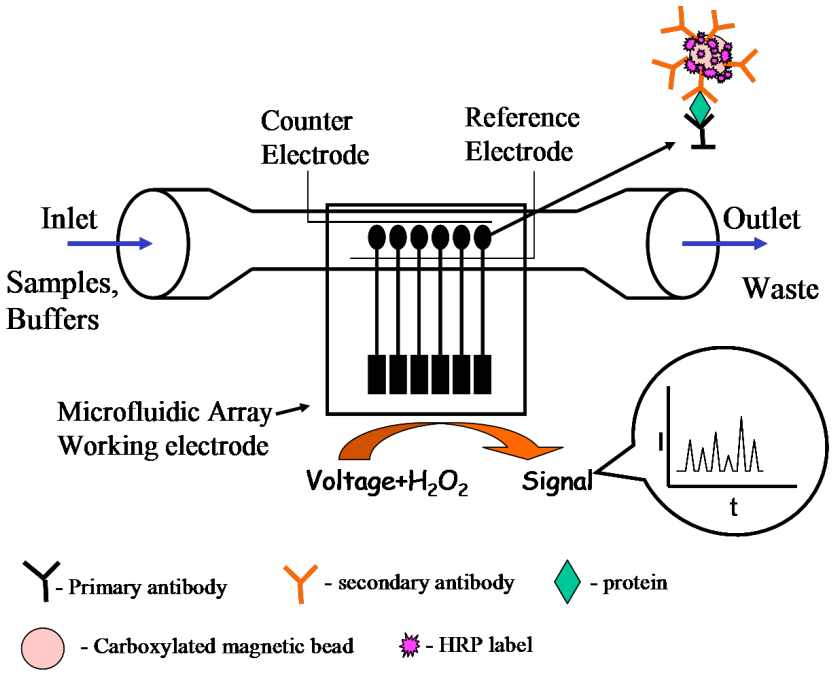

Figure S4. Conceptual diagram of the Microfluidic Immunoarray.

solution was injected into the microfluidic channel and incubated for 10 min followed by washing with 0.05% Tween-20 in PBS and PBS buffer. Then, mixture of MPs labeled with sec. antibodies, HRP with bound PSA and IL6 were pumped into the microfluidic channel. When sample was in the microfluidic channel, as evidenced by the red-brown color of the particles, flow was stopped for a 15 min. incubation. This was followed by washing with 0.05% Tween 20 in PBS and PBS buffer. Amperometric detection was performed at -0.2 V by injecting a mixture of 1mM hydroquinone and 100 µM hydrogen peroxide in PBS buffer at 100 µL min^{-1} flow rate.

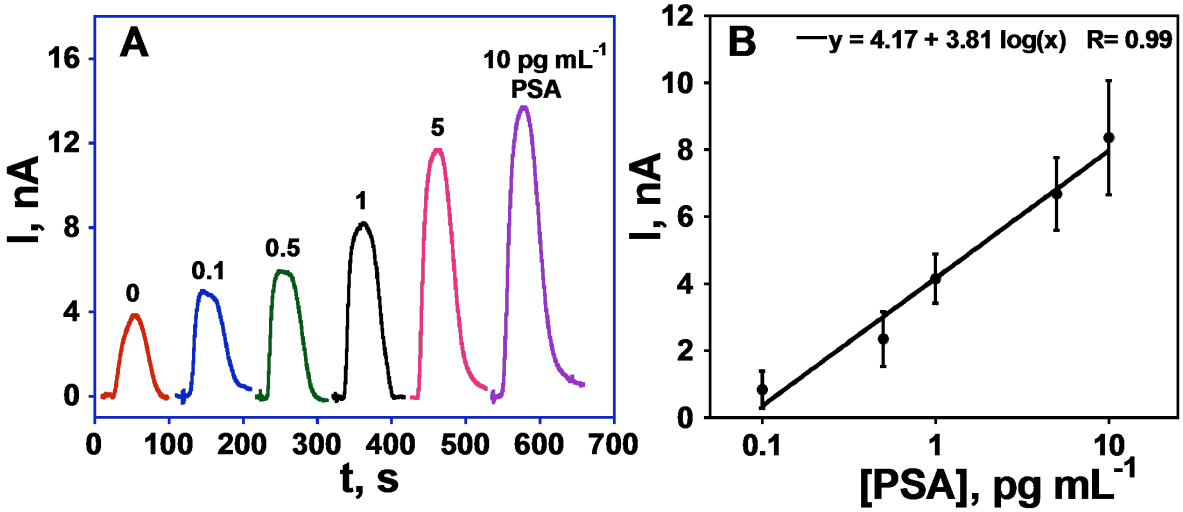

Figure S5. Amperometric responses for PSA alone at -0.2 V developed by injecting a mixed 1 mM hydroquinone and 0.1 mM H_2O_2 solution after capturing analyte protein-MP-Ab_2-HRP bioconjugates on the electrodes in the microfluidic device.

Determination of the cross- reactivity of the analytes:

The immunoarrays were tested for the cross reactivity of the protein with non-specific antibodies before doing detection in mixtures (Figure S6). Amperometric responses for the cross reactivity of the protein with non-specific antibodies is shown in Figure 3. In these experiments, the arrays were coated with both PSA and IL6 primary antibodies, but the MP-Ab_2-HRP-Ag conjugates injected into the microfluidic device were conjugated with only one type of protein. The amperometric measurements were recorded after injecting the mixture of 1 mM hydroquinone and 100 µM H_2O_2. Minimum cross reactivity between the analyte and non specific antibodies was noticed. Amperometric response of the IL6 capture antibodies to 1 pg mL^{-1} PSA conjugated with MP-Ab_2-HRP bioconjugates mixture was 8.3±0.2 nA where as PSA capture antibodies to 1 pg mL^{-1} PSA conjugates gave 13.5±1.1 nA. Amperometric response of the IL6 and PSA capture antibodies coated array to 5 pg mL^{-1} IL6 conjugated with MP-Ab_2-HRP bioconjugates mixture was 16.2 ± 1.3 nA and 9.5 ± 0.2 nA. For the control experiment in which no PSA or IL6 was bound to MP conjugates, the arrays gave 9.2 ± 0.7 nA for IL6 capture antibodies and 7.9 ± 0.9 nA for PSA capture antibodies. Based on t-test, with 99% confidence level, the amperometric responses of PSA and IL6 with non specific antibodies are significantly different. Cross reactivity between PSA with IL-6 capture antibodies was 7 ± 2% whereas the cross reactivity between IL6 with PSA capture antibodies was 4 ± 1%.

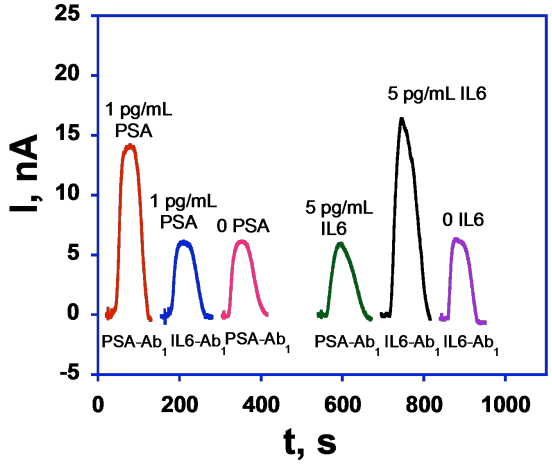

Figure S6. Cross reactivity of the analyte with non specific antibodies. Microfluidic array was coated with both PSA and IL-6 primary antibodies. MP-Ab_2-HRP conjugates were allowed to capture one type of analyte before incubating in the microfluidic device. Amperometric signals recorded to a mixture of buffer containing 1 mM hydroquinone and 0.1 mM H_2O_2.

To test reproducibility, 3 concentrations of the analytes were randomly selected and assay was performed on different days. Figure S7 shows the reproducibility of the amperometric response of 0.5 pg mL^{-1}, 1 pg mL^{-1} and 5 pg mL^{-1} PSA and 1 pg mL^{-1}, 2.5 pg mL^{-1} and 5 pg mL^{-1} IL6 respectively.

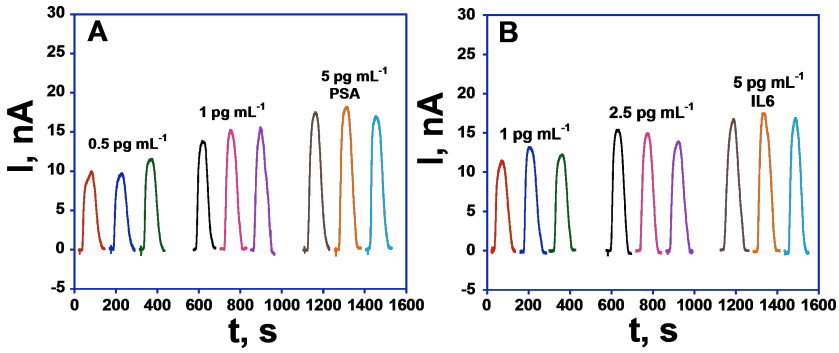

Figure S7. Amperometric responses of the microfluidic array to a mixture of buffer containing 1 mM hydroquinone and 0.1 mM H_2O_2 showing the reproducibility for 3 different concentrations in the low pg mL^{-1} range of (A) PSA and (B) IL-6 on different days under same experimental conditions.

The average standard deviations

were 22% at 0.5 pg mL^{-1}, 11% at 1 pg mL^{-1} and 14% at 5 pg mL^{-1} for PSA and 19% at 1 pg mL^{-1}, 18% at 2.5 pg mL^{-1}, and 15% at 5 pg mL^{-1} for IL-6.

Assay validation with human serum samples. Microfluidic immunoarray results were compared with the ELISA for all the serum samples (Figure S8). Immunosensor results showed a very good correlation with ELISA for all the serum samples with slopes of correlation plots of 1.0 and intercepts of zero, within experimental error (Table S1).

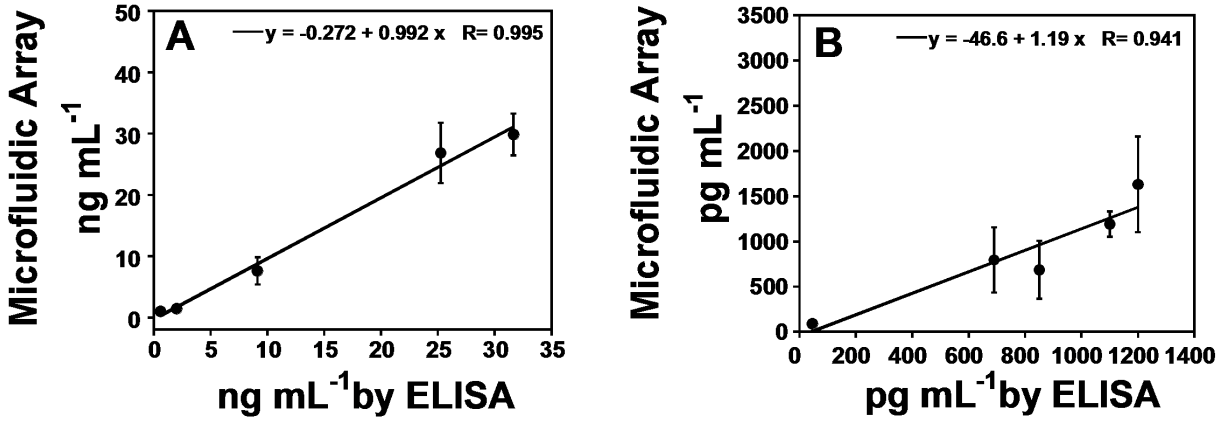

Figure S8. Correlation plots of Microfluidic immunoarray results for human serum samples against results from ELISA determinations for the same samples (A) PSA, (B) IL-6.

TABLE S1. Slopes and Intercepts of Correlation plots of Microfluidic immunoarray results for the human serum samples against results obtained from ELISA determinations for the same serum samples

Biomarker	Slope ± sd	Intercept ± sd
PSA	0.99 ± 0.06	-0.27 ± 1.1
IL-6	1.2 ± 0.25	-46.6 ± 216.7

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