

**An Integrated Approach to a Portable and Low-Cost Immunoassay
for Resource-Poor Settings****

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[**] This research was supported by DARPA/NSF (ECS-0004030) and NIH (GM 65364), and used the MRSEC shared facilities supported by the NSF under Award No. DMR-9809363. V.L. was a recipient of a postdoctoral fellowship from the Swiss National Science Foundation. A.S. was a recipient of a Howard Hughes Medical Institute Predoctoral Fellowship. We thank Tyler Aldredge of the Center for Genomics Research for technical assistance.

The development of technology for use in resource-poor countries encounters a specific type of challenge not ordinarily faced in academic science: the technology must be inexpensive and it must work with minimal infrastructure. This challenge is particularly severe when the problems being solved are, by their nature, ones that require high-technology solutions. In these kinds of problems, the elegance of the solutions must lie in the use of science to guide the assembly of readily available components into a simple, workable, and well-integrated package. In this paper, we describe an integrated approach to a miniaturized immunoassay called a “POCKET immunoassay” (“POCKET” is short for *portable* and *cost-effective*). This immunoassay has, we believe, the potential to be inexpensive and operable with minimal equipment and technical skills, and shows an analytical performance approaching that of enzyme-linked immunosorbent assays (ELISA) performed in the benchtop format in clinical laboratories.

A top priority for improving health in developing countries is technology for simple, affordable diagnosis of infectious diseases.^[1] Immunoassays such as ELISA are the most reliable and widely used methods for detecting antigens and antibodies, but they require expensive and bulky instruments for optical detection, hours of incubation for diffusion-limited reactions on the surface, and many steps of pipetting.^[2, 3] These constraints prevent the use of ELISA in settings that require low-cost or compact equipment, and in environments that lack electricity or trained personnel. One application with these requirements is detection of infectious diseases in the field in developing countries;^[1, 4, 5] other potential uses include point-of-care diagnostics by first responders and in health clinics,^[6] and detection of biological warfare agents in the field.

[2, 7] Immunochromatographic assays (also known as “strip tests” and “lateral flow assays”) are simple to operate, rapid, and commercially available, but they are less sensitive than ELISA, and give primarily yes/no results;^[8] current work focuses on improving its sensitivity and capacity for quantitative analysis.^[8-10] Moreover, although they are less expensive than many tests, they are still too costly for widespread use in developing countries, and for applications that require high-throughput analysis such as screening of blood samples.^[4] As such, an immunoassay that is portable, rapid and simple to operate (like immunochromatographic assays), and that offers parallel, quantitative analysis and a strong, reliable analytical performance (like benchtop ELISA), will be a useful tool of detection in settings for which neither strip tests nor conventional ELISA are appropriate.

We take a comprehensive approach to the design of the assay by miniaturizing and integrating both the immunoassay and the detection device. The immunoassay in this study is performed in an inexpensive, miniaturized platform (made by soft lithography) that is compatible with microfluidics. Microfluidic immunoassays offer several advantages relative to microwell plates, including kinetically rapid reactions at the surface, and the potential for automated fluid delivery and analysis of many samples in parallel.^[11-14] The use of ELISA in microfluidics, however, poses two problems: the generation by enzymes of freely diffusible products makes detection difficult under conditions of continuous flow, and the small cross-sectional path length in microchannels limits the sensitivity of assays using simple optical detection. We address both of these problems in our approach for the immunoassay: instead of enzyme-conjugated secondary

antibodies, we add antibodies conjugated to 10-nm gold colloids, followed by a solution containing silver nitrate and hydroquinone (as reducing agent) (Figure 1). The gold colloids catalyze the reduction of silver ions to silver atoms; in turn, the solid silver catalyzes the further reduction of silver ions.^[15] The resultant silver film, whose opacity is a function of the concentration of the analyte, partially blocks the transmission of light through the transparent polystyrene plate. Because the opaque silver product is attached to the surface, reduction of silver may be an effective method of amplification for microfluidic devices that operate under continuous flow conditions, and may overcome the limitation of a small path length for optical detection of molecules in microchannels.

A detector for use in the field should be compact, low-cost, battery-powered, and if possible, reusable. Ideally, it should operate under different conditions in the field: for example, in direct sunlight. We designed and built a detector that satisfies these requirements: this device measures the transmission of light through the silver film. The detector consisted of an InGaAlP red semiconductor laser diode (654 nm) as the light source, and an optical integrated circuit (IC) (which contains a photodiode, an amplifier and a voltage regulator; it has a peak sensitivity wavelength of 700 nm) as the photodetector. To enhance the utility of the detector in direct sunlight, we used pulse modulation of the optical signal at 1 kHz to filter out noise from ambient light (most of which is at 0 Hz). This feature permitted measurements to be made under ambient light in the laboratory (as were all measurements shown in this report); neither shining a flashlight onto the detector nor bringing the device outdoors in daylight produced a change in the background signal. (We believe that, in the future, other types of

modulation can be used to further increase the signal-to-noise ratio.) The entire detector was powered using a single 9 V battery (for over three hours of continuous usage), making it suitable for transportation and use in the field in conditions without ground electricity. We also connected the optical IC to a liquid crystal display to obviate the need for a multimeter. The components for this reusable and portable detector were bought from commercial vendors for \$45. (See Supporting Information for the details of the circuit design.)^[16]

We first characterized the performance of silver reduction with optical detection by the portable detector by performing an immunoassay of a model antigen in microwell plates. The concentration of rabbit IgG was determined in an immunoassay featuring a series of dilutions that spanned five orders of magnitude in concentrations (Figure 1b). The opacities of the samples were measured by the optical IC and by a benchtop UV-visible absorbance plate reader. For comparison with the data from the plate reader, transmittance values reported by the optical IC were converted to apparent absorbance values (which accounted for both absorption and reflection of the incoming light by the silver film). The optical IC produced readings in excellent agreement with those of the plate reader (correlation coefficient of 0.996). The imperfect agreement between the two measurement methods may have resulted from inhomogeneity of silver deposition.^[16]

We compared the analytical performance of our method of detection (silver reduction with the low-cost, portable detector) to that of ELISA using the most common reporting systems with benchtop plate readers: absorbance, fluorescence, and

chemiluminescence (Figure 2a). For this comparison, we used ELISA substrates that are highly sensitive. We fit the titration data to sigmoidal curves, normalized the best-fit curves of all methods to between 0 and 1, and calculated the sensitivities and limits of detection.^[16] For an immunoassay detecting rabbit IgG, the most to least sensitive methods (as defined by the slope of the titration curve in the middle of the linear range of detection, in normalized units per 100 pM) were: chemiluminescence (0.19), fluorescence (0.12), silver (0.08), and absorbance (0.04). For limits of detection, the assays with the lowest to highest limits were: chemiluminescence (22 pM), absorbance (55 pM), silver (89 pM), and fluorescence (163 pM). Immunoassays using silver reduction showed an average overall standard deviation of 7% (for multiple independent measurements of the same concentration of analyte) when measured by the plate reader, and 13% when measured by the optical IC, compared to 9% to 15% for the ELISA measurements. Thus, the overall analytical performance (sensitivity, limit of detection, and reproducibility) of the POCKET immunoassay in the microwell format approaches that of benchtop ELISA.

We characterized the process of silver deposition by performing an immunoassay for rabbit IgG in microwells, and stopping the silver reduction at various time points. As determined by UV-visible spectroscopy, after incubation with the silver enhancement solution, silver reduction exhibited an initial slow growth phase, and then proceeded rapidly at an approximately linear rate (Figure 2b). Analysis of the same samples by atomic force microscopy (AFM) confirmed that the increase in opacity of the surface correlated with the growth of silver particles (Figure 2b). In our assays, quenching the reaction after 10 to 20 minutes (see Supporting Information for specific times for each

assay) resulted in reproducible amounts of silver that were formed for triplicates in a single experiment. A normalization procedure such as the one used in Fig. 2a (or other methods for running calibration curves) can be used to account for day-to-day fluctuations in the rate of silver deposition (due to an increase in temperature, for example, which increases the rate of silver deposition).

We developed the immunoassay in a microfluidic format by quantifying anti-HIV-1 antibodies in the sera of HIV-1 infected patients (Figure 3). The microfluidic device was fabricated in poly(dimethylsiloxane) using soft lithography.^[17] For comparison, we also performed the same immunoassay in the microwell format. In both formats, the opacity of the silver film was measured using the portable detector. Incubation times required for each reagent were 10 minutes in the microfluidic device, and 1 to 3 hours in the microwells (i.e. a 6- to 18-fold reduction in the time required for this part of the assay). The POCKET immunoassay, in both the microwell and microfluidic format, can reliably distinguish the sera of HIV-1 infected patients from those of non-infected patients (Figure 3). Moreover, the assay in both formats can detect quantitative differences in the amount of anti-gp41 in the sample (Figure 3). At high concentrations of serum (low dilutions), the lower signals of the negative controls in the microfluidic device compared to microwells may have resulted from better washing of antibodies that were non-specifically bound to the surface. We believe that in the future, the pipetting steps of ELISA in microwells can be automated in the microfluidic device, although we have not implemented this feature yet in our device.

This study offers an alternative approach to other efforts for miniaturizing immunoassays for portable use. Methods for analysis of biomolecules on microchips include electrochemical detection,^[12] electrical detection,^[18] and integrated on-chip optical detection.^[7, 19, 20] Compared to the detection of colorimetric and fluorescent substrates in solution, detection of silver reduction catalyzed on gold colloids – a method that has been used in other applications to analyze proteins^[15, 19, 21] and DNA^[22-25] – offers a number of advantages: (i) it is an effective method of signal amplification under conditions of continuous flow in microfluidics; (ii) it circumvents the problem of a small path length in microchannels; (iii) silver film, unlike fluorescent substrates, does not photobleach; (iv) silver, unlike solutions of optically active molecules, is stable for months, which allows results of immunoassays to be kept for long-term use (after 23 days of storage in ambient laboratory conditions, the absorbance readings changed by 1.5%); (v) silver film blocks light at a broad spectrum of wavelengths (the absorbance of silver showed a maximum variation of 20% from 400 nm to 1000 nm), whereas chromophores and fluorophores are active only at specific wavelengths. With silver reduction, a wide variety of laser diodes and photodetectors (of any wavelength) can be used in the detection device.

Overall, the integrated POCKET immunoassay has several advantages: i) it is low-cost and portable, and therefore is appropriate for use in the field; ii) the reporter method of silver reduction is compatible with the use of microfluidics under continuous flow conditions, which decreases the time required for the assay and makes possible a simplified delivery of reagents; iii) the optical detector is battery-powered, reusable, and,

with pulse modulation, operable under field conditions such as direct sunlight; iv) the analytical performance of this integrated miniaturized device approaches that of ELISA using relatively expensive benchtop equipment. The POCKET immunoassay may therefore be appropriate for applications in resource-poor settings, including diagnosis of infectious diseases in developing countries.

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Figure Legends

Figure 1: Schematic representation of the POCKET immunoassay, and performance of the optical detection device. (A) Red light from the laser diode passed through the silver-coated microwell containing the sample to the optical IC. A pinhole was used to block stray light that did not pass through the sample. The laser diode and the optical IC were driven by the same circuit, which also had an integrated liquid crystal display that showed the measured transmittance value. (B) Comparison of readings of an immunoassay using an optical IC and a UV-visible plate reader. An immunoassay using silver reduction was performed on a 96-well plate that detected rabbit IgG. Optical micrographs of the silver films on microwells are shown for each sample. The apparent absorbance of each microwell was measured by an optical IC, and compared to its reading by a UV-visible plate reader; both measurements were made at 654 nm. The best-fit line by linear regression has a correlation coefficient of 0.989, slope of 1.12, and y-intercept of 0.16.

Figure 2: Performance of the POCKET immunoassay. (A) Comparison of the POCKET immunoassay using detection by silver reduction and optical IC with detection by absorbance (pNPP substrate), fluorescence (AttoPhos substrate), and chemiluminescence (Supersignal ELISA Femto, a derivative of luminol, as substrate). Standard deviations of triplicates in a single experiment are shown as error bars. See Supplementary Information for the procedure for normalizing the signal for different assays. In concentration units, 6.7 pM corresponds to 1 ng/mL. (B) Kinetics of silver deposition in

an immunoassay (for all samples, the concentration of rabbit IgG was 67 nM, and the dilution of gold-labeled anti-rabbit IgG was 1:100). The microwells were incubated in silver enhancement solutions for the indicated times, the reaction was quenched with sodium thiosulfate, and the apparent absorbances of the silver films were measured by a UV-visible plate reader. AFM images of samples at three different time points are shown. Standard deviations of triplicates in a single experiment are shown as error bars.

Figure 3: Detection of anti-HIV-1 antibodies in human patient sera using the POCKET immunoassay. (A) Schematic representation of a microfluidic device that detects anti-HIV-1 antibodies. The HIV Env antigen is patterned onto a polystyrene surface as a stripe, and a slab of PDMS with microchannels is placed orthogonally to the stripe. A sequence of reagents is added to the microchannels using pressure-driven flow: blocking buffer, human serum sample, gold-labeled anti-human IgG, and the silver enhancement solution. Analysis of many dilutions was achieved in parallel by adding a different dilution of the human serum sample to each microchannel. (B) Photographs of the detection areas with reduced silver films. (C) Apparent absorbance values from an immunoassay detecting different dilutions of sera from HIV positive patients and control patients. For comparison, the assay was also performed in a 96-well plate. Standard deviations of triplicates in a single experiment are shown as error bars. The apparent absorbance values of the samples shown in part B are shown in this graph.

Suggestion for the Table of Contents

This work describes an integrated approach for detecting proteins in the field. This “POCKET immunoassay” is low-cost, portable, and battery-operated, works under field conditions such as direct sunlight, uses commercially available components, and exhibits an analytical performance approaching that of ELISA using benchtop equipment. It may be appropriate for applications in resource-poor settings, including diagnosis of infectious diseases in developing countries.

Key words: analytical methods, immunoassays, microfluidics, proteins, colloids

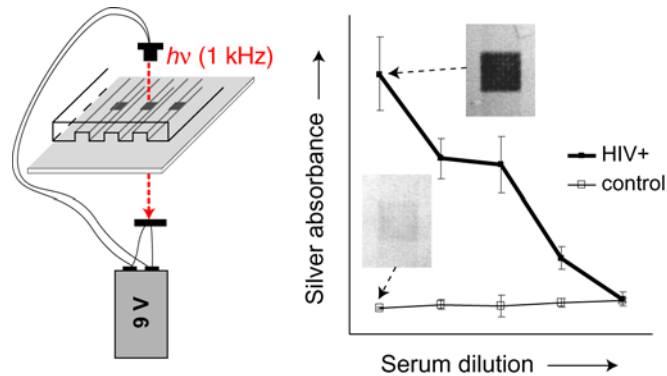


Figure 1

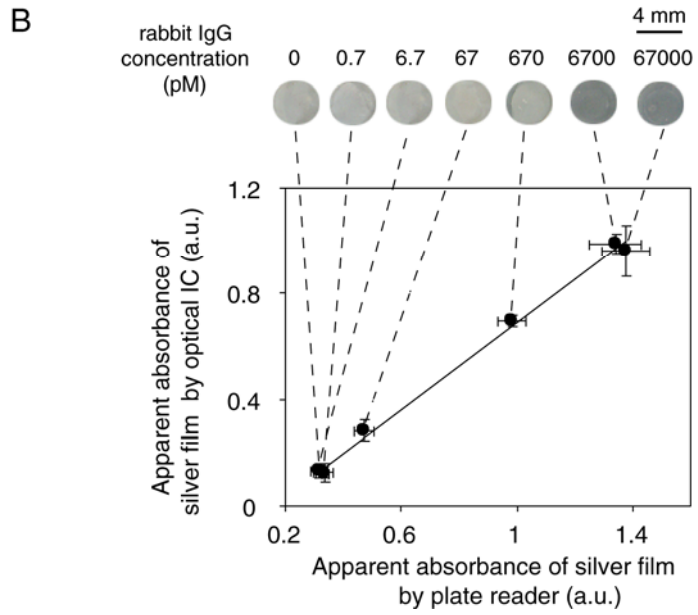
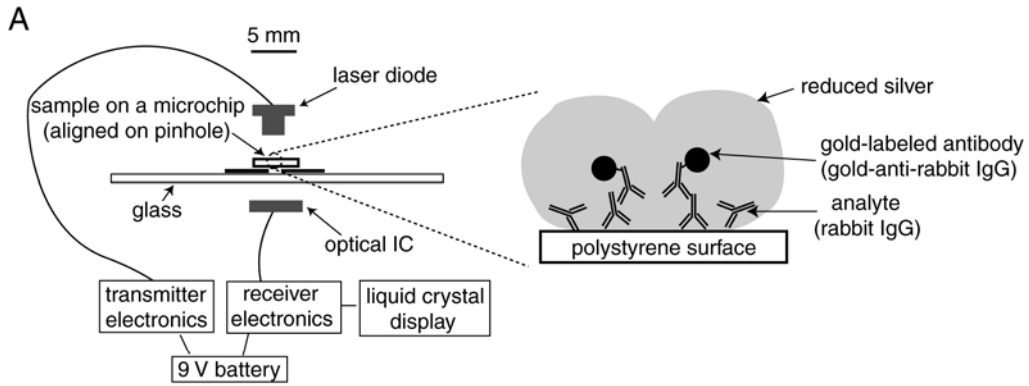


Figure 2

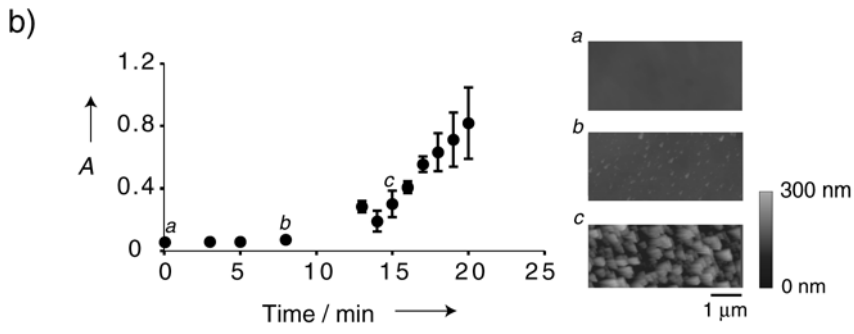
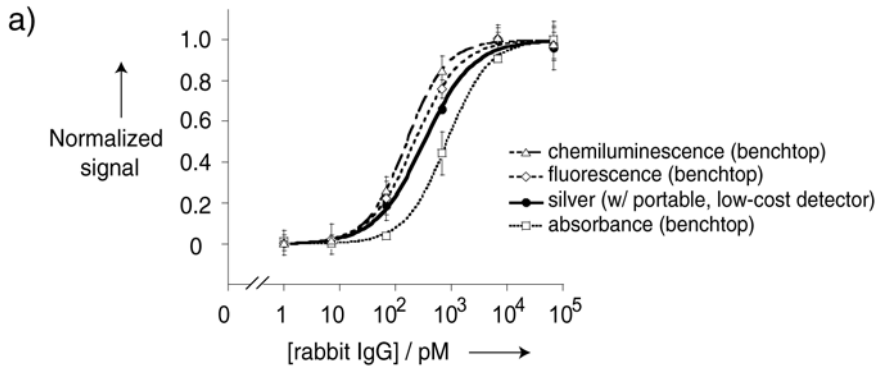
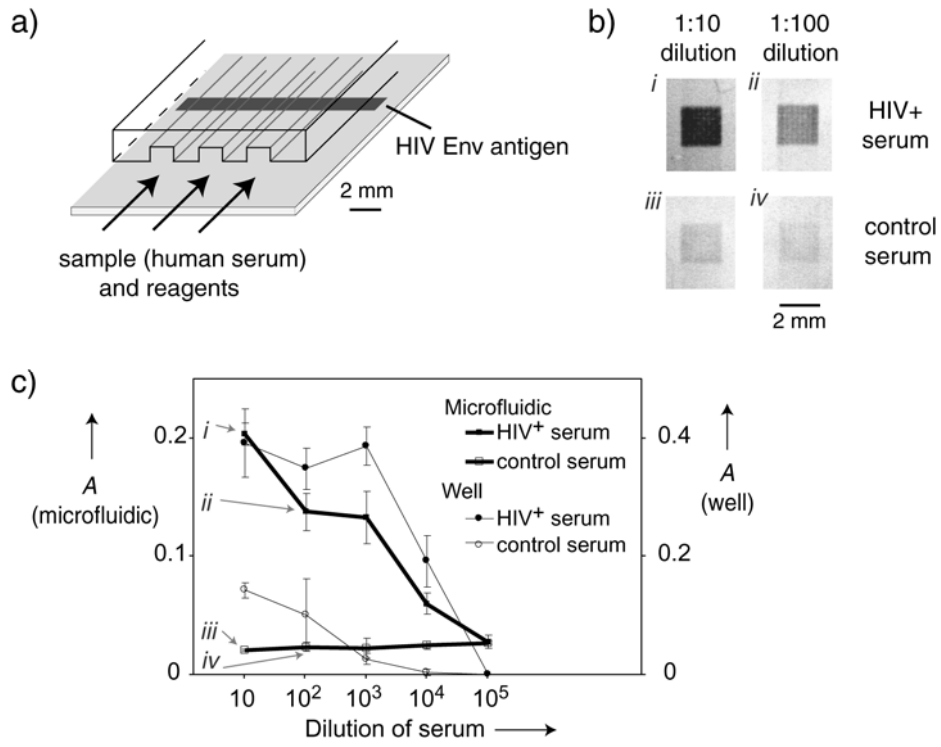


Figure 3



Supporting Information for

**An Integrated Approach to a Portable and Low-Cost Immunoassay
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Supplementary Information

Materials and methods

Rabbit IgG, anti-rabbit IgG (horseradish peroxidase conjugated), anti-rabbit IgG (alkaline phosphatase conjugated), anti-rabbit IgG (gold conjugated), p-nitrophenylphosphate (pNPP), and the silver enhancement kit were obtained from Sigma-Aldrich (St. Louis, MO). AttoPhos was purchased from Promega Corp. (Milwaukee WI). SuperSignal ELISA Femto Max was purchased from Pierce (Rockford, IL). BluePhos phosphatase substrate was purchased from KPL (Gaithersburg, MD). HIV Env antigen (gp41) was purchased from Research Diagnostics (Flanders, NJ). HIV positive serum and control serum were purchased from Golden West Biologicals Inc. (Temecula, CA).

Immunoassays in 96-well microtiter plates were performed using a Tecan Genesis liquid handling robot (Center for Genomics Research, Harvard University). The following Nunc MaxiSorp polystyrene plates were used for the silver reduction and ELISA assays: clear plates for silver reduction and absorbance, black plates for fluorescence and white plates for chemiluminescence. Rabbit IgG (70 μ L for each well) in ten-fold dilutions (10 μ g/mL to 100 pg/mL, which corresponded to 67 nM to 670 fM) was added to the microwells, except for one row to which PBS was added as a negative control; incubation time was 2 hours. Blocking buffer (100 μ L of 0.05% Tween-20 and 1% BSA in PBS) was then added, and left to incubate for 30 minutes. For secondary antibodies, dilutions (30 μ L of 0.05% Tween-20 in PBS) of 1:300 anti-rabbit IgG (gold-

conjugated), 1:1000 anti-rabbit IgG (alkaline phosphatase), and 1:1000 anti-rabbit IgG (horseradish peroxidase) were used; incubation time was 1 hour. For ELISA substrates, pNPP (100 uL; 3 minute incubation), AttoPhos (100 uL, used within 1 week of opening; 10 minute incubation), and SuperSignal Femto ELISA (100 uL; after 5 minutes). For silver enhancement, the solutions of silver and initiator (at 4°C) were mixed in a 1:1 ratio immediately before development; it was filtered through a 0.2 µm filter, and 100 uL was added to each well. After a 20 minute incubation, the silver enhancer solution was removed, and each well was washed with water. In general, warming the silver enhancement solution from 4°C to room temperature increased the rate of silver deposition. In between the addition of each new reagent, each well was washed three times with PBS, with the following exception: deionized water was used to wash the wells after incubation with anti-rabbit IgG (gold) and before silver enhancement, in order to avoid precipitation of AgCl. The plate readers used were Spectramax Plus 384 for absorbance measurements, and Spectramax Gemini XS for fluorescence and chemiluminescence measurements.

The output of the optical IC was light transmittance; apparent absorbance values were calculated using the relation $A = -\log(T / T_0)$, where A is the absorbance, and T and T_0 are the transmission of the light through the sample and reference, respectively, to the photodetector. Air was used as the reference in the plate reader, and a blank polystyrene plate was used as the reference for the portable detector.

The absorbance, fluorescence, and chemiluminescence readings (y) were fit to sigmoidal curves using the software Kaleidagraph and the following equation: $y = Ax^n / (B + x^n) + C$, where x is the concentration of the analyte, and A , B , C and n are floating parameters. This equation describes a general sigmoidal curve with the lowest possible number of floating parameters (four). Curve fitting to all four titrations gave correlation coefficients of over 0.99. The readings y for all four titrations were normalized to the same scale (0 to 1) by linearly transforming each data set to achieve the values of $A = 1$ (asymptote as x approaches infinity) and $C = 0$ (y-intercept).

Limits of detection were calculated according to the IUPAC definition: three times the standard deviation of the blank sample (“noise”) divided by the slope (“sensitivity”). In samples with no rabbit IgG (i.e. negative controls), the methods that exhibited the least to most noise were (after normalization of the signal from 0 to 1): 0.006 for absorbance of pNPP, 0.014 for chemluminescence of SuperSignal ELISA Femto Max, 0.023 for silver (using the portable detector), and 0.066 for fluorescence of AttoPhos. The methods that showed the highest to lowest sensitivities, which were measured as slopes of the best-fit curves in the middle of the linear working range of detection (signal of 0.50), were (in normalized units per 100 pM of analyte): 0.193 for chemiluminescence, 0.121 for fluorescence, 0.078 for silver, and 0.035 for absorbance.

To prepare immunoassay samples for analysis by AFM, holes (4 mm in diameter) were punched in a PDMS slab, and the PDMS slab was placed onto a polystyrene surface. Immunoassays were carried out in individual PDMS wells. After silver

development, the PDMS slab was removed, and the samples on the flat polystyrene substrate were analyzed by tapping mode AFM. AFM was performed with a Dimension 3100 Scanning Probe Microscope (Digital Instruments, Santa Barbara, CA) in tapping mode, using silicon probes (Si #MPP-111000; NanoDevices, Santa Barbara, CA) at a scan rate of 0.35 Hz. Streaking was observed for samples with the largest silver grains, which suggested that the silver grains were loosely bound to the surface.

The microfluidic device was fabricated in PDMS using published procedures in soft lithography^[17]. The dimensions of the microchannels were 2 mm in width and 130 μm in height. We initially patterned the polystyrene surface with a stripe of HIV Env antigen (10 $\mu\text{g}/\text{mL}$) by filling a PDMS channel (conformally sealed onto the polystyrene plate) with the antigen solution. After an overnight incubation, we emptied the channel, removed the PDMS slab from the polystyrene surface, and rinsed the surface with deionized water. We covered the stripe of antigen with an unstructured slab of PDMS, and oxidized the remaining surface of polystyrene with oxygen plasma^[17]. After removal of the plasma-protective PDMS slab, we sealed another microfluidic channel (also freshly plasma-oxidized) orthogonally to the antigen stripe. The dimensions of these microchannels were 2 mm in width and 40 μm in height; the width of the channel must be large enough to register a signal with the portable detector. To avoid sagging of the PDMS, pillars (which took up 12% of the surface area) were included in the channel design. We carried out the anti-HIV antibody assay in the microfluidic channels with the following incubation times: 1 to 4 hours for blocking, 10 minutes for samples, 10 minutes for gold-labelled anti-human IgG, and 13.5 minutes for silver enhancement solution.

After 6.5 minutes of silver enhancement, we exchanged the silver solution with a freshly prepared one. We removed the PDMS microchannel above the initial stripe of antigen before measuring the optical density of the silver film. The HIV assay in microwells were performed with the following incubation times: overnight for HIV Env antigen, 2 hours for blocking, 3 hours for samples, 1 hour for gold-labelled anti-human IgG, and 10 minutes for silver enhancement solution.

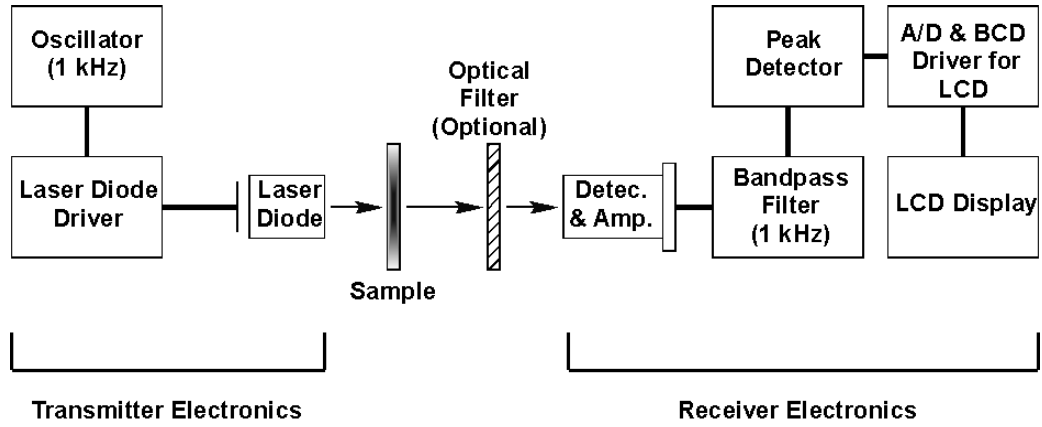
For each concentration of rabbit IgG and each dilution of human serum, triplicates of the immunoassay were performed, and average values and standard deviations were calculated.

The electronic circuit consisted of a transmitter section and a receiver section. In the transmitter section, a 1 kHz oscillator modulated the light output of a laser diode. We used a red semiconductor laser diode (Sharp GH06510B2A; normally used for optical data storage applications such as DVD); it emitted at a wavelength of 654 nm with a maximum power of 10 mW. The laser output went through the sample to the receiver section. We used an optical IC (Sharp IS455; normally used in photocopy machines) to detect and amplify the signal. IS455 provided a linear output current with respect to the input illuminance (1 μ A per lux). (The dimensions and costs of the red laser diode and the optical IC were 5.6 mm and \$10, and 5.0 mm and \$2, respectively.) The signal was then filtered by a second-order bandpass filter centered at 1 kHz, and its amplitude registered by a peak detector. The output of the peak detector was connected to an Analog/Digital converter that also encoded the output into binary coded decimal (Intersil

ICL7106). The signal was displayed by a 3.5 digit liquid crystal display, which provided an output readout range from 0 to 1999. The entire circuitry was operated with either a 9 V battery or a single polarity 5 V source, which was inverted with a CMOS voltage converter (Intersil ICL7660) to create a ± 5 V supply. To reduce the noise in the system, we used pulse modulation of the optical signal at 1 kHz to filter the noise power in the frequency spectrum; as a result, only the portion of the optical noise that fit in the pass band of the receiver filter contributed to the overall noise detected. The system could also function without the signal modulation (i.e. at direct current), albeit with higher sensitivity to optical noise from the surroundings.

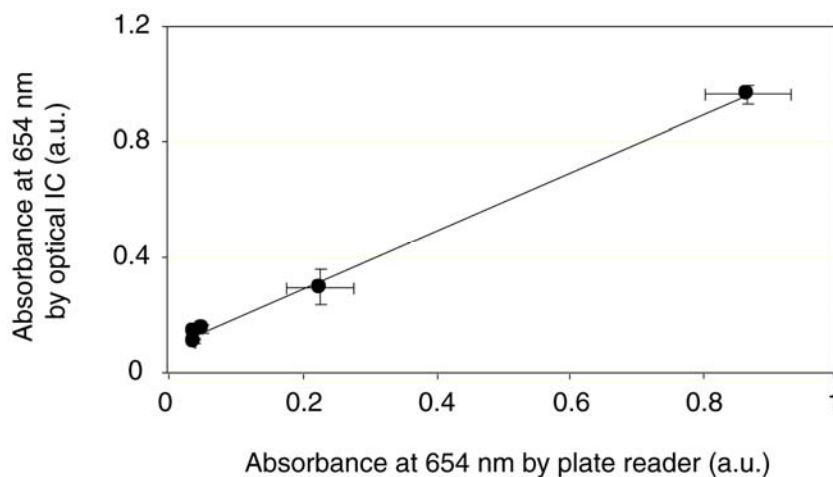
The laser diode and optical IC were placed on two separate circuit boards that were held at a fixed orientation to ensure consistent alignment of the light path from the light source to the photodetector. Between the light source and photodetector, a glass plate was placed. A black transparency, with a pinhole aligned with the light path, was placed on the glass plate to block the transmission of stray light that did not enter the sample. To record a measurement, a polystyrene plate (either a 96-well plate or a plate with a microfluidic device) was placed onto the glass plate. The sample was aligned to the light path by roughly placing the sample over the pinhole, and finely adjusting the x and y position of the polystyrene plate until a maximum transmittance was achieved. The reading from the liquid crystal display was recorded.

Supplementary Figure 1. Optical detection device: schematic of the circuit.



See experimental section for details of the circuit.

Supplementary Figure 2. Comparison of absorbance readings of BluePhos using an optical IC and a UV-visible spectrophotometer plate reader.



Absorbance of microwells containing different concentrations of BluePhos, which absorbs maximally at 600 nm, as measured by a UV-visible absorbance plate reader and the optical IC described in this study. A direct ELISA was performed on 0.67 pM to 0.67 nM of rabbit IgG as the analyte, using an anti-rabbit IgG conjugated to alkaline phosphatase and BluePhos as the phosphatase substrate. Measurements with both devices were made at 654 nm. The best fit line by linear regression is shown (correlation coefficient of 0.998, slope of 1.01, y-intercept of 0.08). Error bars are standard deviations of measurements of three different microwells.

In this assay, in which the colorimetric product is a homogeneous solution in the microwell, the two detection methods resulted in almost perfect agreement (correlation coefficient of 0.998). Thus, inhomogeneity of silver deposition on the surface may have

contributed to the imperfect agreement between the two measurement methods, such that different parts of the same well were sampled by the laser diode and by the plate reader (correlation coefficient of 0.996).