

41 was flowed orthogonally across the stripes of adsorbed antigens, the antibodies in the serum bound to these antigens. The proteins were thus immobilized in a series of rectangular spots, formed by overlapping the antibody and antigen streams. The patterns were then washed by flowing BSA in the fluid paths. The antibodies interacted with the antigens that are adsorbed irreversibly on the membrane in a concentration-dependent manner, the same as in a typical serially diluted immunoassay. The amount of adsorbed anti-GP120 and anti-GP41 in this sample of serum was visualized using a secondary antibody tethered to a fluorophore (anti-human IgG-FITC, FIGS. 24-25). In FIG. 24, each rectangular spot represents a pairwise interaction between an adsorbed antigen and an immobilized antibody from a solution of different concentration (decreasing from left to right, with a serum free control on the far right). The fluorescent signals of the distinct micropatterns for different antigens bound to different amounts of antibodies indicate the concentrations of the antibody in the solution.

[0128] FIG. 25 shows the results from three independent assays. The error bars show one standard deviation from the mean of fluorescence intensities. From these assays, it may be inferred that the patient's serum has antibodies that have high affinities to the antigens presented on the polycarbonate surface. The serial dilution experiments show that the serum is positive for both anti GP 120 and GP 41: fluorescent signals decreased rapidly in a concentration-dependent manner between fluid paths 8 and 10 of the system of FIG. 24, while keeping at a low level in fluid paths 10 and 11. Fluid path 12 is the negative control; its fluorescent signal is lower than the lowest dilutions, since in fluid paths 1-11 there are some non-specific IgGs bound to the substrate. The difference between the signals in fluid path 12 and the average signals in fluid paths 10 and 11 shows the strength of this background binding, caused by non-specific IgGs (IgGs that do not bind specifically to GP 120 or GP 41) in the serum. Between fluid paths 8 and 10, the area where the titration between the antibody and antigen is carried out, the signal decreases in a concentration-dependent manner. Before fluid path 8, the fluorescent signal is saturated for both anti GP 120 and anti GP 41. After fluid path 10, the signal no longer decreases, meaning that after this dilution, the antibodies no longer react specifically. Had such a wide dynamic range not been scanned, it would not have been possible to see a concentration-dependent change in the antibody-antigen reactions, and one might have been lead to think that either the interaction was non-specific (in the range between fluid paths 1 and 7) or there was no reaction at all (in the range between fluid paths 10 and 12).

[0129] In this assay, flowing liquids were used to ensure proper mixing inside the fluid paths. This assay differed from flow-injection assays, in that the flow speed that was typically very low (approximately 3 nL/s or 0.6 cm/s in a fluid path with dimension of approximately 100  $\mu\text{m}$   $\times$  50  $\mu\text{m}$ ), compared with most flow-based assays (ranging from 100 nL/s to 10<sup>5</sup> nL/s). Accordingly, this assay mimics traditional immunoassays in 96-wells. The same experiment was repeated with an additional incubation step (1 hour at 37° C.) where the antibodies (serum containing anti-GP 120 and anti-GP 41) were allowed to react with the antigens after the serially-diluted antibodies had been flowed in, (mimicking the incubation step done in microtiter plates in traditional 96-well plate). Less than 7% deviations in fluorescent intensities across all of the fluid paths were observed. The

additional incubation step was possible because the individual fluid paths were physically separate and the diluted antibody-containing solutions from each fluid path did not mix with each other. Flows of serum and BSA sustained for 15 min-4 hours (at 3 nL/s) gave similar immobilization results, with or without a subsequent incubation step of 1 hour at room temperature.

[0130] In traditional HIV testing, positive serum reactions for any one type of antibody is typically not definitive evidence for HIV infection. Additional tests such as serum reactions to other kinds of HIV viral antigens, or Western blots are needed to confirm initial results. These experiments demonstrate that a single device according to the present invention can eliminate some uncertainties in determining HIV infection, since reactions to multiple viral antigens can be determined in a single experiment. The experiments further demonstrate that the methodology of the present invention provides a general route to quantifying the interactions between one molecule and many other molecules on the surface at the same time. Quantitative assays of multiple analytes simultaneous with very small volumes of liquid of this format may find wide uses in clinical, pharmaceutical and environmental sciences.

#### Example 5

[0131] In order to demonstrate that the method and apparatus of the invention may also be adapted for use in inhibition assays, such an assay was attempted. In an inhibition assay, an inhibitor that prevents binding between an antibody in solution and an antigen immobilized on a substrate is serially diluted. Using an inhibition assay it was possible to obtain the binding constant between the antibody in the solution and the antigen, as well as the concentration of the antibody in the solution.

[0132] The inhibitor (which was the same molecule as the antigen on the surface) was flowed instead of the dilution buffer into the inlet, with the rest of the experiment the same as described in Example 4. The serial dilution generated a gradient of inhibitor. Since the inhibitor competes with the surface-immobilized antigen to bind the antibody in the solution, different amounts of immobilization of the antibody on the surface was achieved. Then by assaying the amount of antibody immobilization from a second antibody that is fluorescently labeled, it was possible to determine the amount of antibody immobilization as a function of the inhibitor concentration. From the curve plotted between the fluorescence intensity (which is a measurement of the amount of immobilized antibody) and the concentration of the antigen (which was known), it was possible to obtain the binding constant between the inhibitor (antigen) with the antibody in mol/L. Performing this kind of assay in microfluidic fluid paths not only saves the volume of solution by orders of magnitude, but also enables assaying for two or more kinds of antibody-antigen interactions quantitatively in one experiment. This demonstrates that the method and apparatus of the invention may be adapted for use in inhibition assays.

[0133] Two different microfluidic devices were fabricated to determine the efficacy of the devices for a titration. Materials and reagents for Examples 6 and 7 were obtained or fabricated as described below.

[0134] Microfluidic devices were fabricated as follows: Sylgard® 184 Silicone, a two-part poly(dimethylsiloxane)