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Integrated Microfluidic Device for Serum Biomarker Quantitation using Either Standard Addition or a Calibration Curve

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Abstract

Detection and accurate quantitation of biomarkers such as alpha-fetoprotein (AFP) can be a key aspect of early stage cancer diagnosis. Microfluidic devices provide attractive analysis capabilities, including low sample and reagent consumption, as well as short assay times. However, to date microfluidic analyzers have relied exclusively on calibration curves for sample quantitation, which can be problematic for complex mixtures such as human serum. We have fabricated integrated polymer microfluidic systems that can quantitatively determine fluorescently labeled AFP in human serum, using either the method of standard addition or a calibration curve. Our microdevices couple an immunoaffinity purification step with rapid microchip electrophoresis separation with laser-induced fluorescence detection system, all under automated voltage control in a miniaturized polymer microchip. In conjunction with laser-induced fluorescence detection, these systems can quantify AFP at ~1 ng/mL levels in ~10 μL of human serum in a few tens of minutes. Our polymer microdevices have been applied in determining AFP in spiked serum samples. These integrated microsystems offer excellent potential for rapid, simple and accurate biomarker quantitation in a point-of-care setting.

The two most widely used quantitation tools in traditional analytical chemistry are the calibration curve and the method of standard addition.[1] Micromachined devices for chemical analysis[2, 3] that integrate multiple processes,[4] reduce sample and reagent consumption,[5] and decrease analysis time[6, 7] and instrument footprint,[8, 9] are becoming an attractive alternative to classical separation-based analysis approaches. Although calibration curves have been used in microchip-based chemical analysis,[10, 11] the method of standard addition, which is especially desirable for addressing matrix effects in complex samples[1] such as blood, has seen extremely limited use. Very recently, a serial dilution microfluidic device was applied in standard addition quantitation of mM concentrations of $Fe(CN)_6^{4-}$, a model analyte, although the aqueous KCl solution was not one for which matrix effects were anticipated.[12]

Alpha-fetoprotein (AFP) is a diagnostic biomarker for *Hepatocellular carcinoma* (HCC),[13] with a reported specificity of 65% to 94%.[14] In general, patients with an elevated serum AFP concentration have a higher risk for HCC. Currently, enzyme linked immunosorbent assay (ELISA) is used in the clinical analysis of AFP in human serum.[15] With trained personnel, ELISA can provide reliable results, although the multi-hour assay times and microplate format make ELISA best suited for clinical, rather than point-of-care (POC) diagnostics. In contrast, rapid analysis[6, 7] and the ability to combine multiple processing steps[4, 16] on a single device make a microfluidic-based approach very attractive for POC AFP analysis. The analysis and separation of AFP in spiked buffer solutions in a microdevice platform have been reported,[17–19] and chip-based microfluidic assay systems for other analytes have been developed for

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saliva[10] and blood samples.[11, 20, 21] However, only calibration curve quantitation has been explored.

Our work relies on microfluidic immunoaffinity extraction, which is illustrated in Fig. 1. Antibodies are immobilized on a patterned section of a microchannel surface to form an affinity column. When a sample flows through the column, only antigen will be retained based on antibody-antigen interaction, while non-target material will pass through the column to waste. This approach has been shown to capture target proteins from buffer solutions[22] in a microdevice, but the ability to work with complex specimens such as blood, and integrate capture with separation[23] has not been shown.

Here, we demonstrate an integrated microfluidic system capable of performing quantitative determination of AFP, a biomarker for liver cancer,[24] in human serum, using both the method of standard addition and a calibration curve. Our approach utilizes an immunoaffinity purification step coupled with rapid microchip electrophoresis separation, all under voltage control, in a miniaturized polymer microchip. These systems with laser-induced fluorescence (LIF) detection can quantify AFP at ~1 ng/mL levels in ~10 μL of human serum in a few tens of minutes, offering exciting potential for POC applications.

EXPERIMENTAL SECTION

Affinity column formation

A prepolymer mixture containing glycidyl methacrylate as the functional monomer, poly (ethylene glycol) diacrylate (575 Da average molecular weight) as the crosslinker, and 2,2-dimethoxy-2-phenylacetophenone as the photoinitiator was prepared. Before polymerization, the mixture was sonicated in a water bath for 1 min, followed by nitrogen purging for 3 min to remove dissolved oxygen. The degassed mixture (10 μL) was pipetted into reservoir G (Fig. 2a), filling the microchannel via capillary action. Next, vacuum was applied to reservoir G to remove most of the monomer solution, leaving a coating of the prepolymer mixture on the channel walls. The microchip was covered with an aluminum photomask with a 4×4 mm^2 opening to provide spatial control of polymerization. The microchip was then placed on a copper plate in an icebath, and exposed to UV light (200 mW/cm^2) in the wavelength range of 320–390 nm for 5 min (cooling helped minimize undesired thermal polymerization). Finally, any unpolymerized material was removed by flushing 2-propanol through the microchannels using a syringe pump.

Fluorescently tagged sample preparation

A 3-mL aliquot of fresh human blood was obtained from a healthy volunteer in a 4-mL Vacutainer tube (BD) at the Brigham Young University Student Health Center. The blood sample was centrifuged at 5,000 rpm (Eppendorf 5415C) for 10 min to separate the serum from whole blood. FITC and Alexa Fluor 488 TFP Ester (Invitrogen) were used to label amino acids, proteins, and serum samples using protocols provided by Invitrogen (MP 00143). Briefly, 0.1 mg fluorescent dye was dissolved in 10 μL DMSO. For amino acid or protein standards, a 5-μL aliquot of this DMSO solution was immediately mixed with 0.2 mL of sample (1 mg/mL) in 10 mM carbonate buffer (pH 9.0). For serum samples, a 2-μL aliquot of DMSO solution with dissolved dye was mixed directly with 98 μL of human serum. The mixture was incubated in the dark at room temperature for 24 h (FITC) or 15 min (Alexa Fluor 488). In direct labeling of complex biological specimens, it is essential to have excess dye to ensure complete labeling.

Data analysis

The calculation of AFP concentration was based on the peak heights in the electropherograms both for calibration curve and standard addition methods. For the calibration curve, the AFP

peak height from each standard electropherogram was plotted against the AFP standard concentration to generate a linear calibration curve by the method of least squares. The AFP concentration in the sample was obtained from the electropherogram peak height and the calibration curve. The standard addition method, which effectively eliminates matrix effects, [1] was also used to analyze the AFP samples. Indeed, our protocol of loading sample plus standard on the affinity column is microfluidically equivalent to spiking standards into a sample in a classical standard addition analysis. Peak heights from the electropherograms of the unknown sample, as well as those of the sample plus added standard, were plotted vs. concentration of added standard. The slope and intercept of this line were calculated by least squares analysis, and the unknown AFP concentration was given by the intercept divided by the slope.[1] Standard deviations were calculated from the regression data.

RESULTS AND DISCUSSION

We used a photo-defined immunoaffinity column in a polymeric microdevice to extract AFP from blood serum. Retained AFP was eluted through an injection cross and rapidly analyzed by microchip electrophoresis. To quantify the serum AFP concentration precisely, both standard addition and calibration curve functions were integrated into the chip. Importantly, all fluid control on-chip was carried out via voltages applied to reservoirs, facilitating automation. The fabrication protocol for poly(methyl methacrylate) (PMMA) microdevices, which entailed hot embossing and thermal annealing, was adapted from our previous work. [23, 25] The layout of our integrated AFP analysis microchip is shown in Fig. 2a, and a device photograph can be seen in Fig. 2b. PMMA itself is relatively inert toward direct chemical reaction, which necessitates making a photo-defined polymer on the microchannel surface to immobilize antibodies. The thickness of the reactive polymer formed on the channel surface was ~3 μm. To provide analyte specificity, reactive polymer coated microchannels were derivatized with monoclonal anti-AFP according to our previously described procedure.[23]

To quantify the AFP concentration in serum samples, both calibration curve and standard addition methods were used to validate the accuracy and precision of microchip performance. The voltage configurations and flow paths during operation of the microchip (described below) are shown in Fig. 3. For the calibration curve, each AFP standard solution was loaded on the affinity column for 5 min by applying voltage between either reservoir D, E, or F and reservoir H; the column was rinsed with PBS buffer for 5 min by applying a potential between reservoirs B and H; analyte was eluted/injected with a voltage applied to reservoir J while grounding reservoirs C and G for 45 s using phosphoric acid/dihydrogen phosphate solution at pH 2.1; and then loaded material was separated by microchip electrophoresis using a potential between reservoirs I and L. The sample was analyzed by loading on the affinity column for 5 min with voltage applied between reservoirs A and H, and then rinsing, elution/injection and separation were done the same as with the standards. For the standard addition method, after loading sample on the affinity column for 5 min as above, one standard was loaded on the affinity column for 5 min as before, followed by rinsing, elution/injection and microchip electrophoresis separation, the same as for the calibration curve. This process was then repeated for each standard. LIF was used to detect the labeled AFP during microchip electrophoresis. [26] We note that miniaturized (shoebox size) LIF systems for microchip electrophoresis have been made,[27] indicating their suitability for POC assays.

We characterized the loading, rinsing, and elution profile of fluorescein-5-isothiocyanate (FITC) labeled AFP flowing out from an anti-AFP column. A fluorescence video image (Movie.wmv, Supporting Information) taken after the end of the affinity column shows the retention, rinsing, and elution steps for FITC-AFP.

To demonstrate the integration of immunoaffinity extraction with microchip electrophoresis on a microdevice, a mixture of non-target fluorescent compounds along with FITC-AFP was loaded through an affinity column and then analyzed. Five peaks were observed before extraction, as shown in Fig. S1a; we note that FITC-BSA and FITC-AFP have similar elution times, and are not baseline resolved in the electropherogram. Contrastingly, after on-chip affinity purification (Fig. S1b), all non-target peaks are essentially eliminated, while only the AFP peak remains. Importantly, similar device performance was observed with a much more complex, fluorescently labeled human serum sample. Microchip electrophoresis of FITC-tagged human serum (Fig. 4a) showed numerous overlapping peaks before extraction, precluding facile AFP determination. On the other hand, after on-chip AFP extraction, a single, clear peak corresponding to AFP was observed in microchip electrophoresis (Fig. 4b). The integrated immunoaffinity extraction step resulted in a ~5,000-fold reduction of non-target protein signal, and enabled detection of the AFP "needle" in the serum "haystack". We estimate that the AFP sample is >95% pure after immunoaffinity extraction, based on target to spurious peak ratios in the electropherograms in Figure 4–Figure 5. These results clearly indicate that our approach can selectively purify target analytes from very complex mixtures. A typical affinity column can perform well for at least a few tens of replicate runs.

FITC is a commonly used fluorescent dye for labeling amine-containing compounds such as proteins; however, the room-temperature reaction kinetics (~24 h), make this label less desirable for POC work. On the other hand, we found that Alexa Fluor 488 TFP Ester (Invitrogen) completely labeled AFP in ~30 min (Fig. S2 and Table S1), making this dye very well suited for POC work. In addition, for some microchip bioassays, sample and standards share the same reservoir,[10, 28] requiring a cleaning step during analysis, which hampers the ability to automate for POC assays. In our design, sample and standard reservoirs are integrated on the microdevices. Finally, although previous systems have only used calibration curves to quantify biomarkers,[10, 11] our format enables both standard addition and calibration curve protocols to be performed on-chip.

We used our integrated microdevices to quantify AFP concentration in human serum using either a linear calibration curve (Fig. 5a, 5c) or the standard addition method (Fig. 5b, 5d). Both approaches yielded reproducible microchip electrophoresis data (Fig. 5a, 5b) with concentration-dependent peak heights (Fig. 5c, 5d). AFP concentrations and standard deviations determined both by calibration curve (4.1±0.9 ng/mL) and standard addition methods (4.6±0.9 ng/mL) were internally consistent. To further evaluate our approach, different amounts of AFP were spiked into human serum, and these samples were then labeled with Alexa Fluor 488 TFP Ester. In either calibration curve or standard addition protocols, the standard concentration should be close to the sample concentration for optimal accuracy and precision. However, in POC screening the AFP concentration is initially unknown. Because the action threshold for serum AFP is 20 ng/mL,[15, 29] we set the standard concentrations to 5, 10 and 20 ng/mL in our protocol for optimal precision in the diagnostic range. The AFP concentrations measured in our microdevices using both calibration curve and standard addition methods were compared with values measured by a commercial ELISA kit (Fig. 6). In general, both calibration curve and standard addition results matched ELISA results well (Fig. 6 and Table 1). Because the AFP standard concentrations were optimized for the 20 ng/mL diagnostic threshold, higher AFP concentrations (>50 ng/mL) had lower accuracy and precision; however, a POC assay that reports a concentration well above the action level would require more thorough subsequent clinical analysis.

Although our microdevices have been designed for AFP analysis, this approach is not limited to just AFP. These microchips could be easily adapted for detection of other biomarkers by simply immobilizing different antibodies in the affinity column. Moreover, it should be possible to attach multiple antibodies targeting different analytes to the same column, allowing

multiplexed, simultaneous biomarker detection. Our system shows great promise for rapid quantitation of biomarkers in a POC setting, which should be of considerable value in early stage disease diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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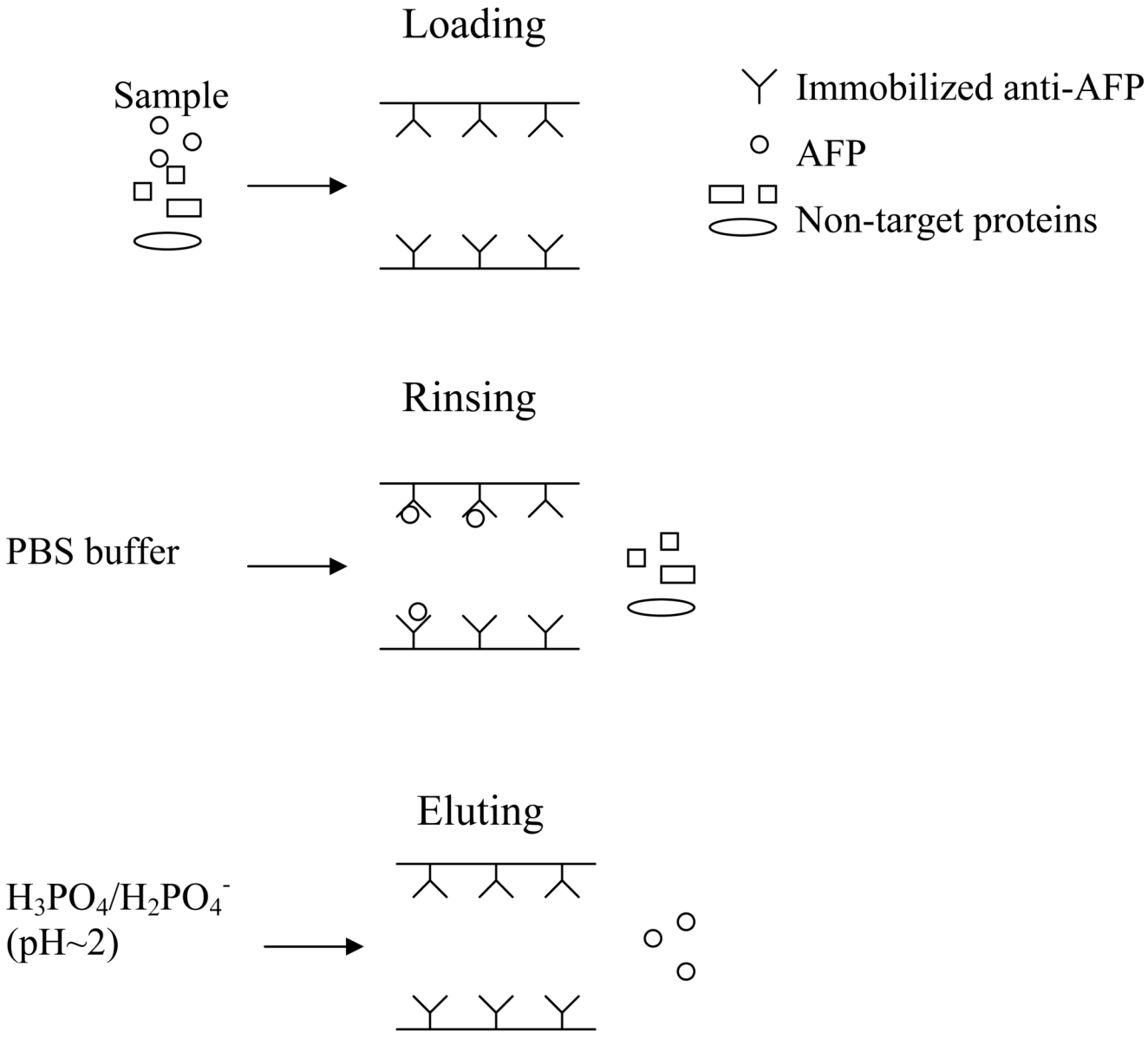

Figure 1.
Immunoaffinity extraction overview.

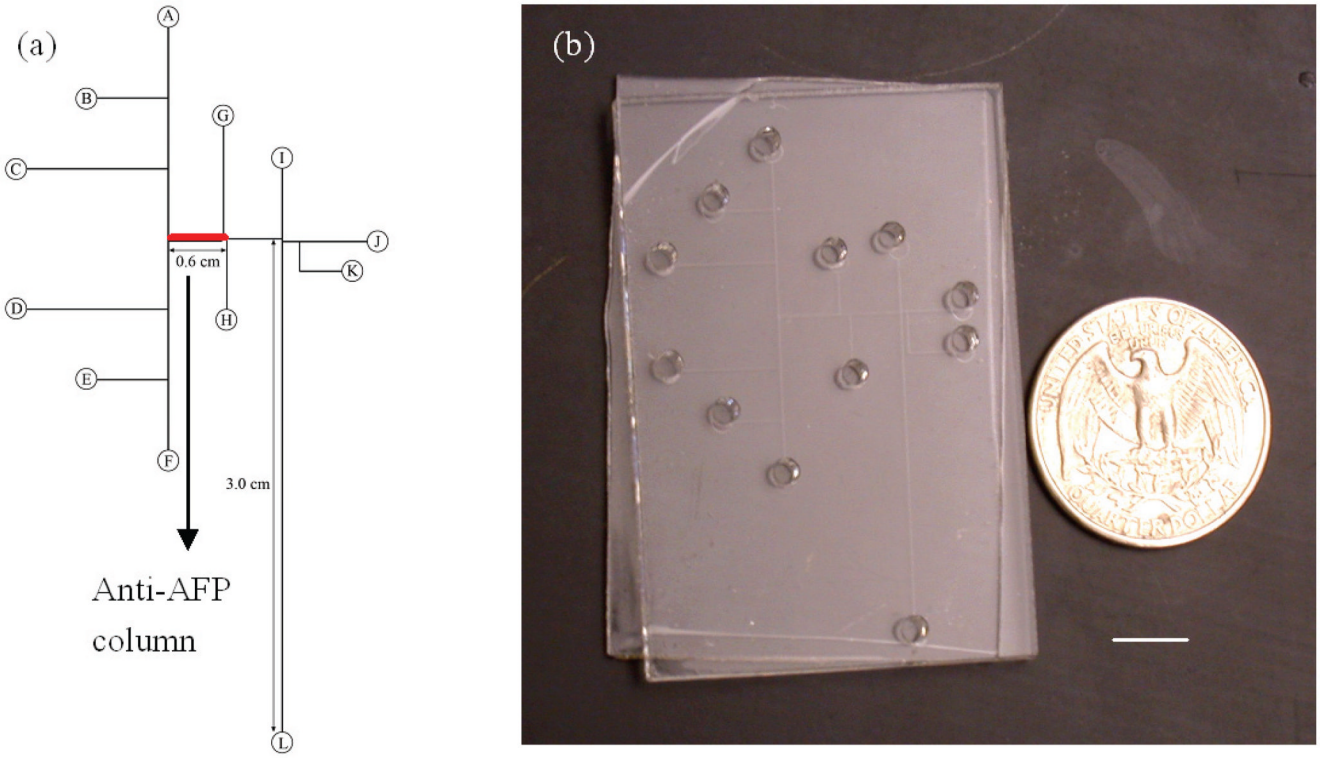

Figure 2.

Layout of an integrated AFP analysis microchip. (a) Diagram and (b) photograph of a microfluidic device with integrated affinity column. Reservoir labels are A: sample, B: rinse buffer, C: elution solution, D: 5 ng/mL AFP standard solution, E: 10 ng/mL AFP standard solution, F: 20 ng/mL AFP standard solution, G: 5 mM NaOH (to neutralize the acidic elution solution during injection), H: waste, and I–L: electrophoresis buffer. Scale bar in (b) is 1 cm.

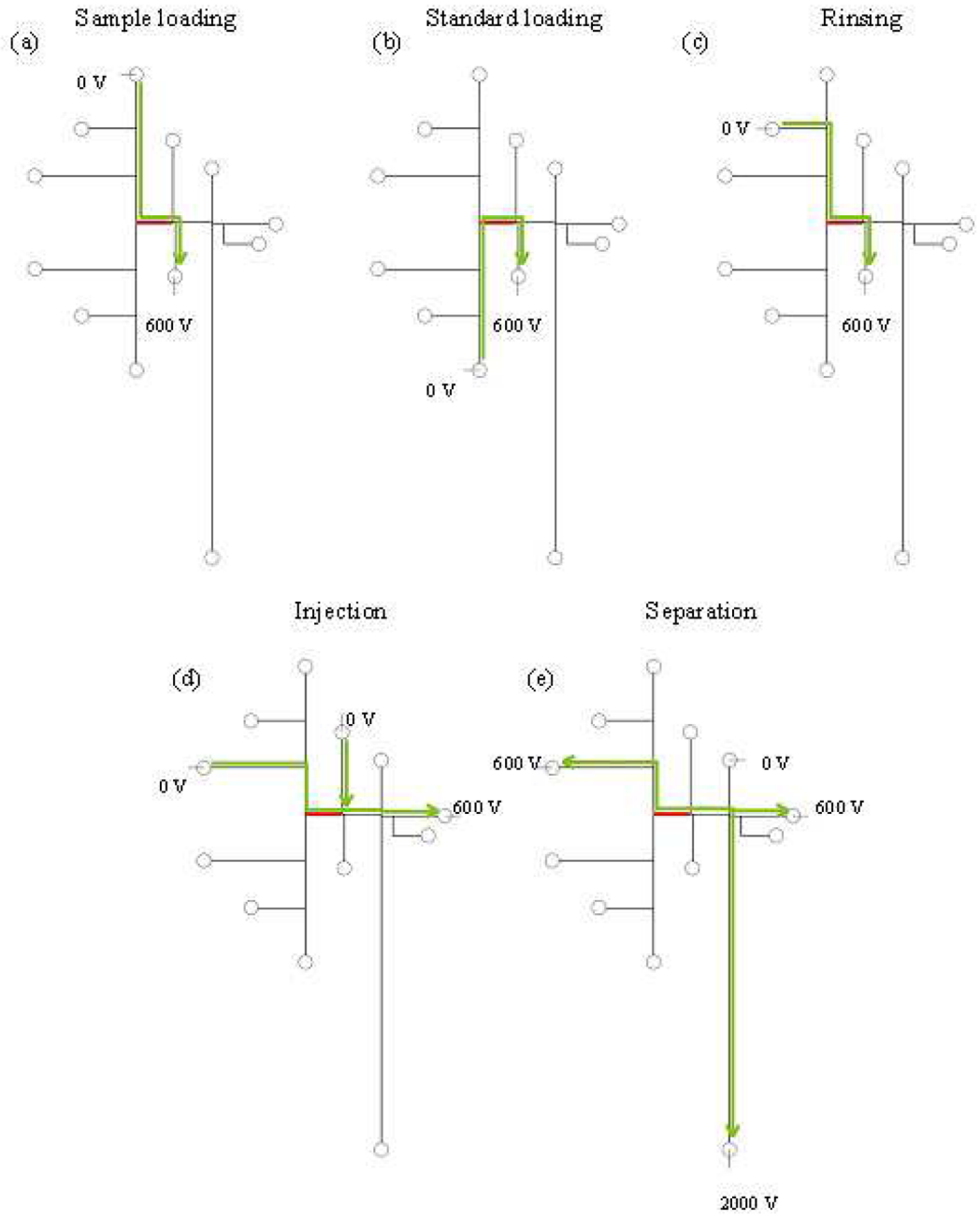

Figure 3.
Schematic diagram of operation of the microchip with integrated affinity column. (a) Sample loading, (b) standard loading, (c) rinsing, (d) injection, and (e) separation.

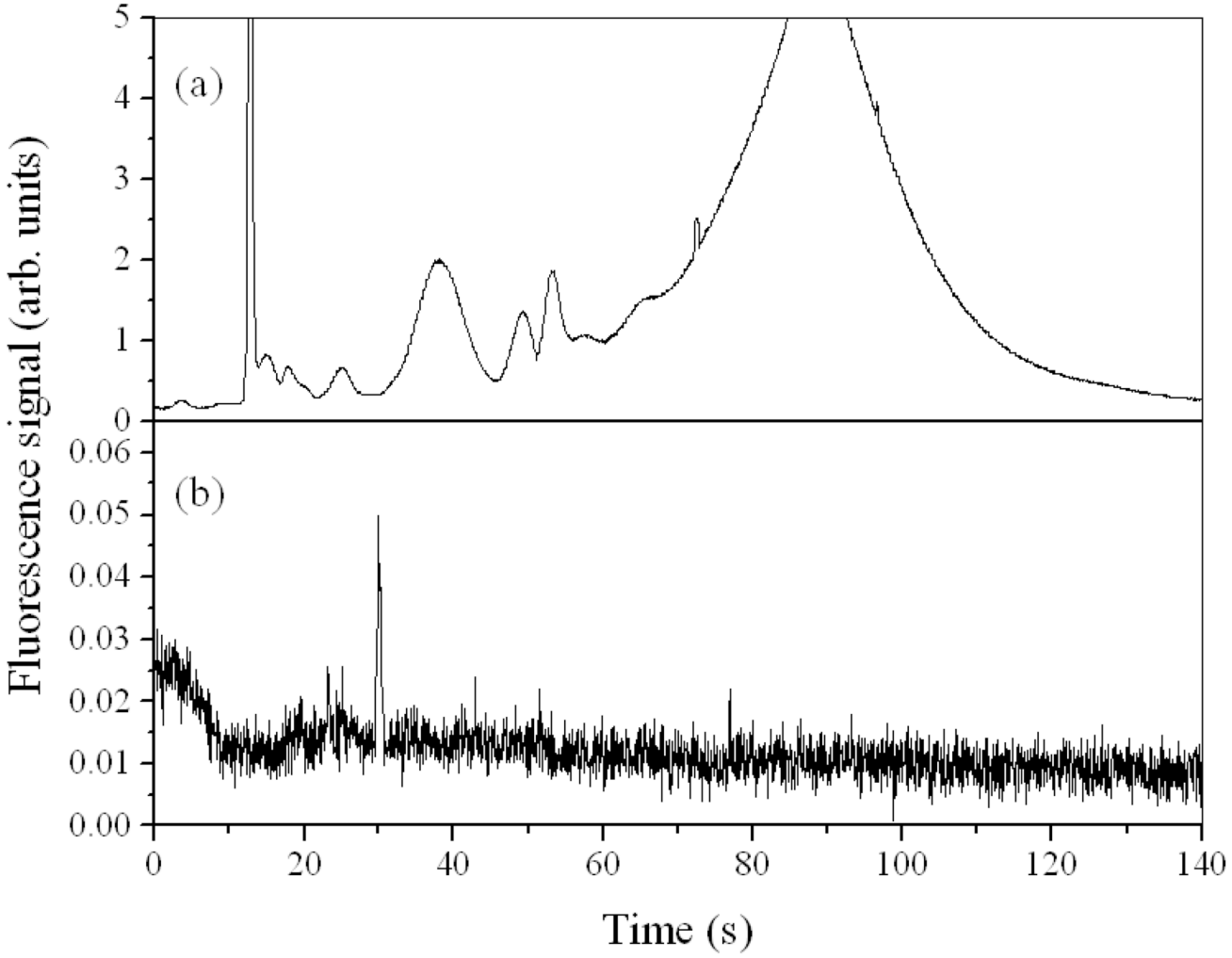

Figure 4.
FITC-labeled human serum, run by microchip electrophoresis (a) before and (b) after integrated affinity column extraction.

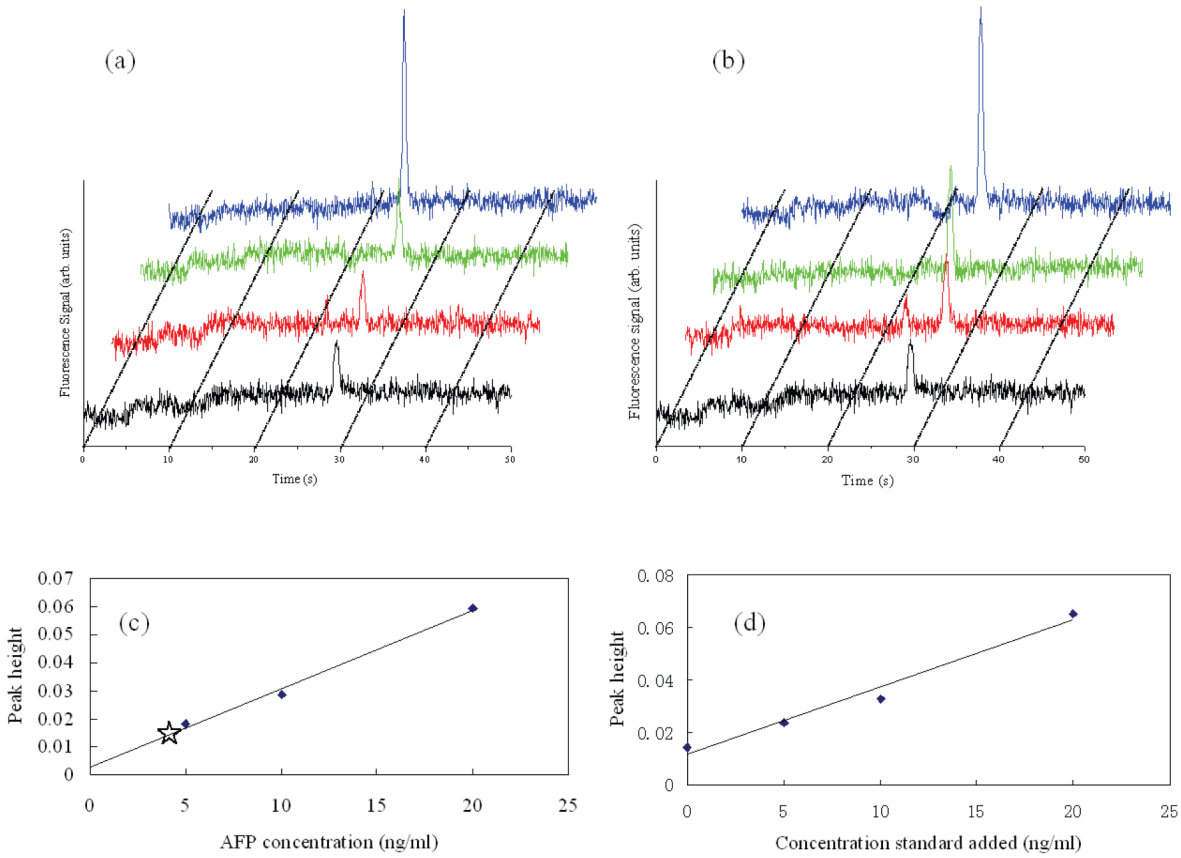

Figure 5.

Integrated calibration curve and standard addition quantification of AFP in human serum. (a) Microchip electrophoresis of Alexa Fluor 488 labeled human serum and of AFP standard solutions after affinity column extraction. Curves are: black—unknown human serum sample, red—5 ng/mL standard AFP, green—10 ng/mL standard AFP, and blue—20 ng/mL standard AFP. (b) Microchip electrophoresis of Alexa Fluor 488 labeled human serum after standard addition and affinity column extraction. Traces are: black—sample, red—sample+5 ng/mL standard AFP, green—sample+10 ng/mL standard AFP, and blue—sample+20 ng/mL standard AFP. (c) Calibration curve generated from (a), with unknown sample data point indicated with a star. (d) Standard addition plot of concentration of standard added vs. peak height generated from (b).

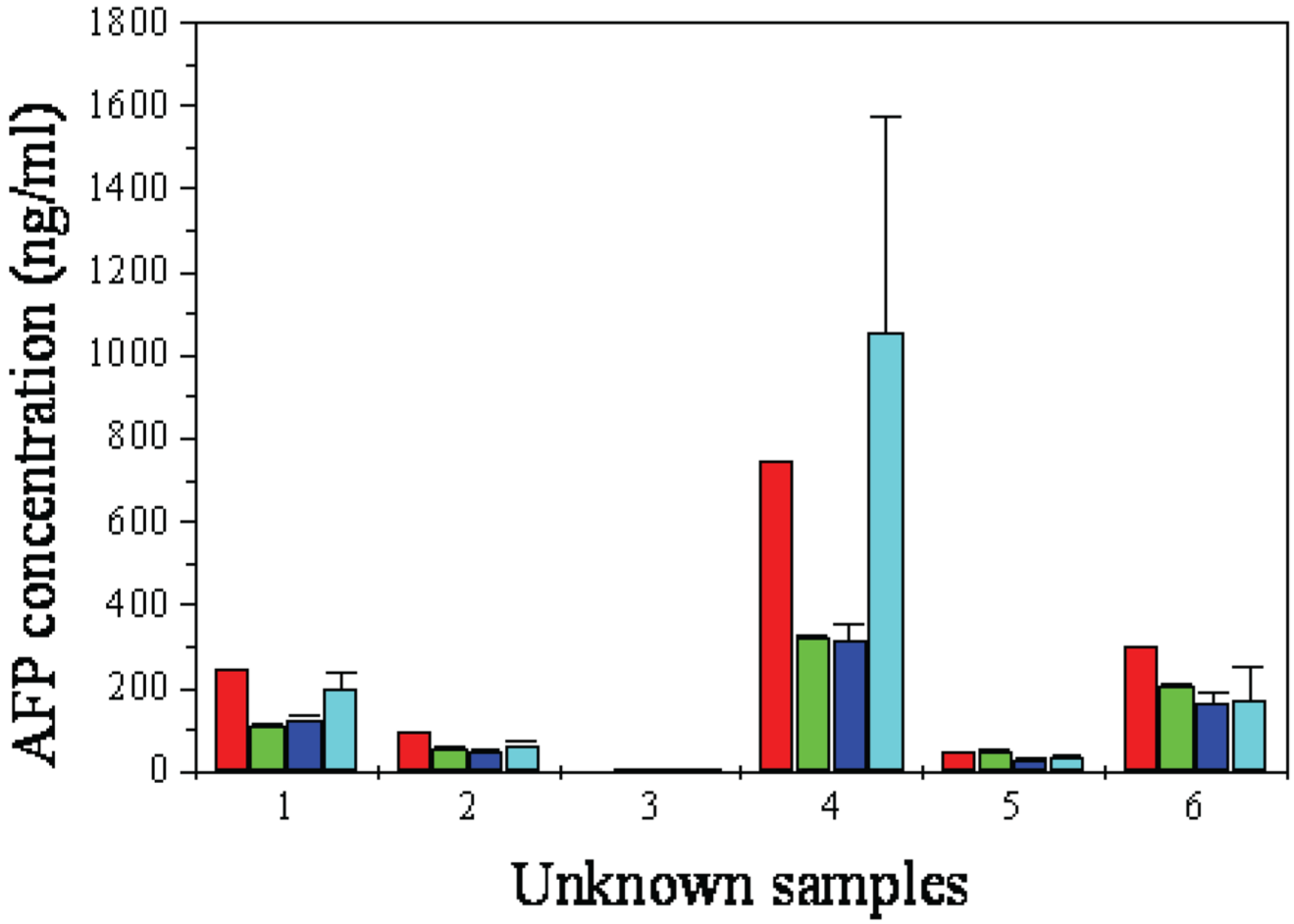

Figure 6.
Accuracy and precision data for integrated microfluidic AFP assay. Red: spiked concentration, green: measured by ELISA (United Biotech, Mountain View, CA), blue: measured by calibration curve, and light blue: measured by standard addition. Error bars indicate ± one standard deviation.

Table 1

Results from the integrated microfluidic AFP assay (the number that follows the ± sign is the standard deviation).

	Spiked AFP (ng/mL)	ELISA (ng/mL)	Calibration curve (ng/mL)	Standard Addition (ng/mL)
unknown 1	250	110.4±2.7	126±6.8	198±41
unknown 2	100	55±2.1	52±1.1	64±8.8
unknown 3	0	2.8±2.0	4.1±0.9	4.6±0.9
unknown 4	750	323.6±6.7	313±41	1050±520
unknown 5	50	49.3±2.0	29.4±0.1	33.2±2.7
unknown 6	300	205.1±4.3	165±25	169±82