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## Development of an automated on-chip bead-based ELISA platform

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### ABSTRACT

We present a lab-on-a-chip and associated instrument for heterogeneous enzyme-linked immunosorbent assay (ELISA)-based detection of proteins from liquid samples. The system performs all necessary ELISA steps (starting from antigen incubation) in a quarter of the time required for corresponding plate-based protocols. We have previously described the instrument, which automates fluidic control via remote valve switching and detects fluorescence from reacted substrate, for use in a molecular diagnostics application. The ELISA chip reported here utilizes a high surface area bead bed to enhance capture efficiency and increase the dynamic range of the assay as compared to a standard plate-based ELISA. Its functionality is demonstrated using human IL-10 as a model antigen, but theoretically any sandwich ELISA could be ported onto this “open source platform.” We show that our automated on-chip assays have greater sensitivities than the corresponding standard manual plate-based ELISAs, and that single samples can be assayed in a fraction of the time.

### INTRODUCTION

The rapid detection of protein analytes from patient samples is critical for proper diagnosis of numerous diseases. Many analytes can be selectively targeted with specific antibodies via enzyme-linked immunosorbent assays (ELISA), an assay format widely used by both research and clinical laboratories.<sup>1</sup> In clinical laboratories, ELISAs are typically run in “batched” format with multiple samples tested simultaneously on one plate. Manual ELISAs require significant hands-on time for sample loading, reagent addition/incubation, washing, and detection, and consistent handling by a skilled operator is requisite for reliable assay performance. Though automated, plate-based ELISA platforms exist, these are expensive and bulky and still require batching of samples. The need for rapid diagnosis to facilitate clinical decision-making has led to the development of simple immunoassays utilizing lateral flow chromatography, which satisfy the goal of reducing results turn-around time for individual patients but typically have low sensitivities and offer only qualitative results.<sup>2</sup> Microfluidic platforms are conducive to random access rather than batched processing, thus reducing results turn-around time. Additionally, they offer the potential for increased sensitivity and quantitation.

Recently, scientists have been porting immunoassays onto novel microfluidic devices.<sup>3,4</sup> Microfluidic ELISA platforms can use optical,<sup>5,6</sup> electrochemical,<sup>7,8</sup> or mechanical read-outs.<sup>4</sup> Antibodies can be attached to the surfaces of the microfluidic channels,<sup>9</sup> linked to beads for increased surface area (magnetic<sup>10,11</sup> or otherwise<sup>12,13,14</sup>), or present in solution.<sup>15,16</sup> Finally,

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3 fluids can be moved either actively,<sup>11,17,18</sup> or passively.<sup>19,20</sup> Aside from the obvious cost  
4 advantages of consuming less of each reagent, using smaller volumes can accelerate the binding  
5 reactions by minimizing the distance needed for analyte/reagent diffusion, and likewise can  
6 increase the sensitivity of an assay.<sup>21</sup> These systems have excellent features including high  
7 sensitivities and short assay times. However, these microfluidic ELISA systems are most often  
8 designed for a single purpose, and multi-functionality is necessarily excluded.

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11 In contrast, we incorporated significant flexibility into our lab-on-a-chip platform design to  
12 ensure that it was capable of automating diverse assays. The instrument has five reagent inputs,  
13 two waste ports, and a bubble drain. Plastic chips (5.3 x 8.5 cm) are seamlessly and  
14 automatically mated to the instrument through a weighted interface block, removing all active  
15 components from the chip. Because water is inherently incompressible, valves on the instrument  
16 can be used to designate various fluid paths through the chip. An on-board spectrophotometer  
17 gives additional flexibility. A previously reported application that utilizes this instrument  
18 involves lysing bacteria, capturing, purifying and eluting their DNA, mixing it with PCR  
19 reagents, thermocycling the mixture in a PCR chamber, and detecting fluorescent product  
20 downstream.<sup>22</sup> Here, we report the adaptation of our fully-automated lab-on-a-chip instrument  
21 for the ELISA-based detection of proteins from liquid samples (Fig. 1a), demonstrating that this  
22 single platform is capable of performing both high-sensitivity immunoassays and nucleic acid  
23 assays on low-cost-to-manufacture microfluidic chips.

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28 We have designed a novel chip and a new pumping program to give maximum flexibility to our  
29 ELISA system. The assay-specific reagents are on-chip (Fig. 1b) and assay-independent reagents  
30 are on the instrument (Fig. 1c), making this platform a valuable resource for laboratories wishing  
31 to run a variety of immunoassays. The planar, credit card-sized, valve-less chip can be injection  
32 molded, and contains a bed of functionalized polystyrene beads that acts as a capture column  
33 (Fig. 1d). The sample reservoir accepts 0.01–1 mL volumes, making it possible—when the  
34 sample is not volume-limited—to measure low concentration analytes without needing to pre-  
35 concentrate the sample. The instrument automates control of the fluids and measures reacted  
36 substrate downstream of the bead bed via on-board fluorescence detection.<sup>22</sup> Using human IL-10  
37 as an example antigen, we show that our automated on-chip assays have comparable sensitivities  
38 to corresponding standard manual ELISAs that take at least four times longer per sample.

## 39 40 41 42 43 MATERIALS AND METHODS

### 44 45 *Chip preparation and run protocol*

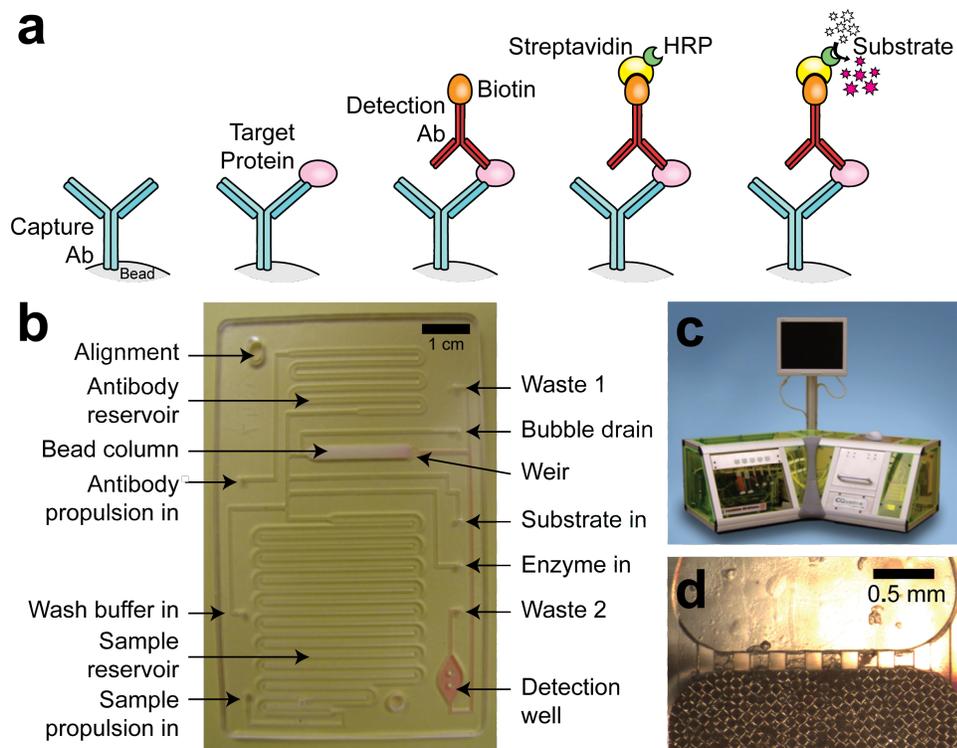
46 The prototype chip features (Fig. 1b) were machined with a computer numerical controlled  
47 (CNC) milling machine in Zeonex<sup>®</sup> 690R, obtained as molded plaques from Zeon Chemicals  
48 (Louisville, KY). Channels were then sealed with a 100  $\mu\text{m}$ -thick cover slip of the same material  
49 using previously described methods.<sup>22</sup> The reusable chips were blocked overnight with a 1:1  
50 mixture of 2% Bovine Serum Albumin (BSA) in water and BlockIt buffer from ArrayIt  
51 Corporation (Sunnyvale, CA). A batch of polystyrene beads (125 mg; 90  $\mu\text{m}$ ; Polysciences, Inc.,  
52 Warrington, PA),<sup>23</sup> sufficient for four assays, was functionalized by incubation with capture  
53 antibody (1 mL Phosphate Buffered Saline [PBS] containing 100  $\mu\text{g}$  mouse anti-human IL-10  
54 from PeproTech, Rocky Hill, NJ) overnight, blocked with 1% BSA in PBS (blocking buffer) for  
55 2 h, and stored at 4 °C in PBS containing *ca.* 0.3% BSA for no more than a week. Immediately  
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prior to each run, beads in storage buffer were pneumatically loaded into the *ca.* 50- $\mu$ L capture column at 50 PSI to ensure a densely packed, immobile bead bed (surface area  $\sim$ 18.5 cm<sup>2</sup>). Chips were then dried with air before being loaded onto the instrument.

Four solutions were used for the on-chip protocol: 1) wash buffer (0.05% Tween-20 in PBS) to prime channels and wash the column, 2) biotinylated detection antibody (biotinylated rabbit anti-human IL-10, Peprotech) at a concentration of 0.25  $\mu$ g/mL in blocking buffer, 3) enzyme (ImmunoPure Streptavidin-Horse Radish Peroxidase [HRP] conjugate from Thermo Fisher Scientific, Waltham, MA) at 1  $\mu$ g/mL in blocking buffer, and 4) fluorogenic substrate (QuantaRed, Thermo Fisher Scientific).

The lab-on-a-chip platform (Fig. 1c) was designed to automate the following steps:

1. load liquid sample (containing human IL-10 from PeproTech) from chip sample reservoir onto column of beads containing capture antibody,
2. wash column to remove unbound sample,
3. load biotinylated detection antibody from chip antibody reservoir,
4. wash column to remove excess antibody,
5. load enzyme from on-board syringe,
6. wash column to remove excess enzyme,
7. load HRP substrate from on-board syringe, incubate on column for 30 sec, and push to detection well,
8. detect reacted substrate with an end-point fluorescence measurement.



**Fig. 1** On-chip bead-based ELISA overview. a: Sandwich ELISA steps performed by our lab-on-a-chip instrument. b: ELISA chip with all inlets, outlet and reservoirs labeled. Note the bead column and reacted

substrate in the detection well. c: Lab-on-a-chip instrument with syringe pumps, pneumatic dispensers, valves and optical detection. d: Microscopic view of the bead bed held in place by the weir.

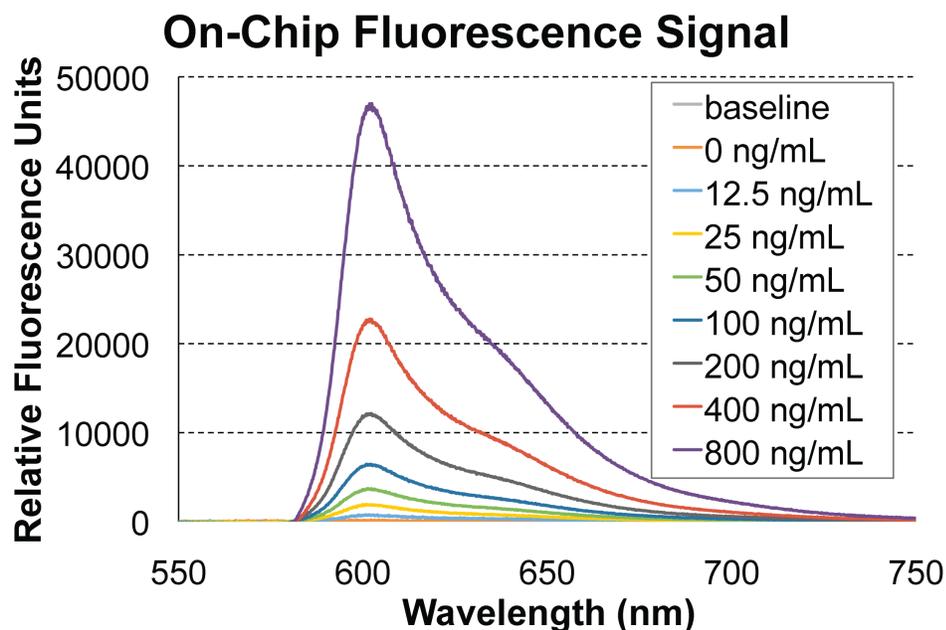
Human IL-10 was diluted 1:1 over a range of 3–800 ng/mL in diluent (0.05% Tween-20, 0.1% BSA in PBS). The sample (up to 1000  $\mu$ L) and the detection antibody (100  $\mu$ L) were manually loaded into separate reservoirs on the chip prior to the start of the run. These were then automatically pushed over the column during the assay with wash buffer as a propulsion buffer, delivered via syringe pumps located on the instrument. The enzyme and substrate were connected to the instrument via 1-mL syringes and delivered by the instrument with pneumatic dispensers. Sample was incubated on the column for 30 min, detection antibody for 20 min, enzyme for 90 s, and substrate for 30 s (Table 1). Total (automated/hands-off) assay time, including washes, was 75 min.

Following QuantaRed incubation, the on-board optical system excited the detection well with 518-nm light and measured the resulting fluorescent signal between 600 nm and 700 nm (Fig. 2). An initial baseline spectrum, which contained signals from stray light, excitation light, and auto-fluorescence of the chip, was taken before the substrate was loaded onto the column. Then, the reacted substrate was pushed into the detection well, and the assay spectra were taken immediately. The reported result is the maximum obtained when the baseline spectrum is subtracted from the assay spectrum.

Using traditional ELISA, the antibody manufacturer (Peprotech) has tested the cross-reactivity of the antibodies against human IL-10 analogues and against rat and murine interleukins IL-4 and IL-10. In all cases, less than 1% cross-reactivity was observed. It is known that “regardless of the format, the specificity of an immunoassay is dependent on the reaction between antibody and antigen.”<sup>24</sup> As such, we have not performed additional specificity testing of our platform.

**Table 1.** Volumes and times used for each step of the on-chip ELISA and off-chip controls

Assay Step	On-Chip ELISA		Standard ELISA		Speed ELISA	
	Volume (mL)	Time (min)	Volume (mL)	Time (min)	Volume (mL)	Time (min)
Load/incubate sample	1	30	0.1	120	0.1	30
Wash	1	7.5	4 x 0.3	10	4 x 0.3	10
Load/incubate detection antibody	0.1	20	0.1	120	0.1	20
Wash	0.8	6.4	4 x 0.3	10	4 x 0.3	10
Load/incubate enzyme	0.14	1.5	0.1	30	0.1	1.5
Wash	2	3.3	4 x 0.3	10	4 x 0.3	10
Load substrate	0.14	0.5	0.1	0.5	0.1	0.5
Detect	On-board		SpectraMax M3		SpectraMax M3	
Total Time	1.25 h		5 h		1.25 h	



**Fig. 2** On-chip detection of various concentrations of human IL-10. Spectra were taken with the on-board optical detection system. Baseline fluorescence signals have been subtracted.

#### *Off-chip ELISA controls*

For the off-chip ELISAs, black Microfluoar 2-coated 96-well polystyrene plates were used (Cat# 7805, Thermo Scientific, Waltham, MA). Wells were coated overnight at room temperature with 0.1  $\mu\text{g}$  capture antibody in 100  $\mu\text{L}$  PBS. The concentration was chosen so that the antibody density was similar between the on- and off-chip assays; that is, the amount of antibody available for the given polystyrene surface area ( $\sim 0.95 \text{ cm}^2$ ) was equivalent to the ratio used for the bead functionalization protocol. The wells were washed four times with 300  $\mu\text{L}$  of wash buffer, and blocked with 300  $\mu\text{L}$  of blocking buffer for 1 h at RT. One part IL-10 was diluted with two parts diluent over a range of 0.15–1000 ng/mL, added to the washed plate (100  $\mu\text{L}$ ), and incubated for 2 h at RT. Detection antibody (25 ng in 100  $\mu\text{L}$  diluent) was incubated for 2 h at RT. Following washing, 100  $\mu\text{L}$ /well of enzyme at a concentration of 1  $\mu\text{g}/\text{mL}$  were added and incubated for 30 min. The plate was washed twice with wash buffer and twice with PBS. After addition of the fluorogenic HRP substrate solution (100  $\mu\text{L}$ /well), the HRP activity was detected by measuring the fluorescence at an excitation wavelength of 530 nm and emission wavelength scan from 600 nm to 620 nm on a SpectraMax M3 (Molecular Devices, Sunnyvale, CA).

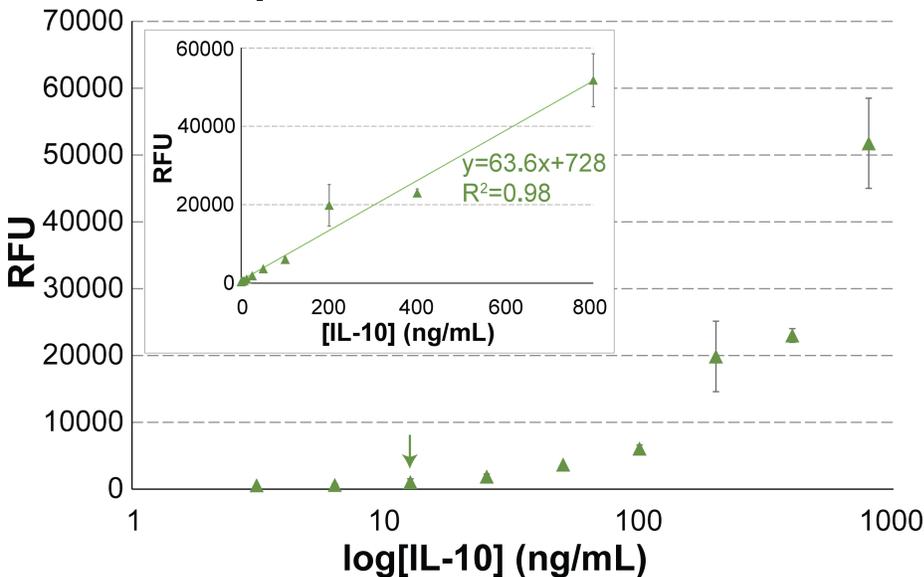
The speed ELISA was performed in the same manner as described above except that the incubation times for the sample, the detection antibody and the enzyme were changed to match the on-chip protocol (see Table 1).

## RESULTS AND DISCUSSION

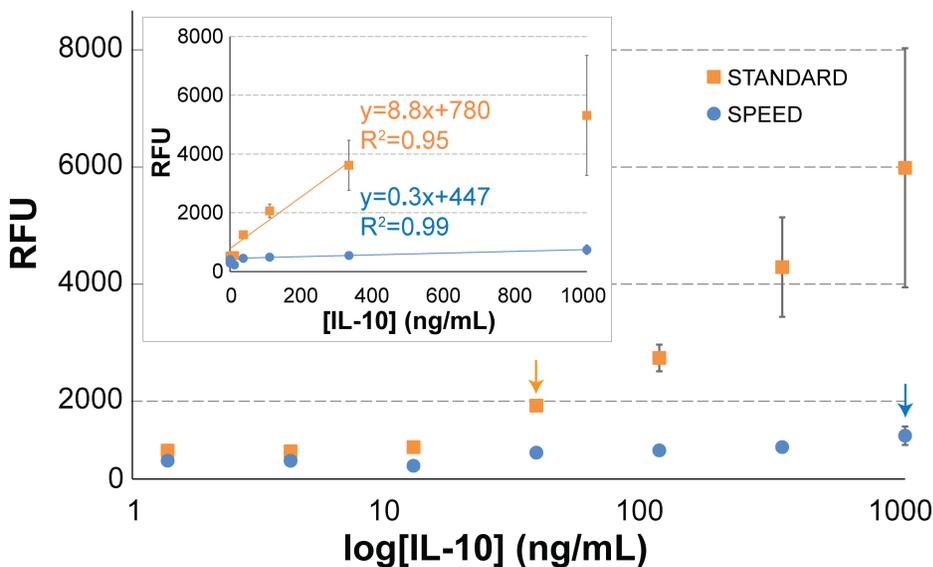
Three chips were used at random to test human IL-10 at concentrations ranging from 3.1 to 800 ng/mL. It was not possible to test higher antigen concentrations under the presented conditions because the resulting fluorescence exceeded the upper limit of the on-board detector.

The limit of detection (LOD; defined here as the antigen concentration that gives a signal three times greater than the standard deviation of the negative control) for our on-chip assay was 12.5 ng/mL (Fig. 3a). According to the manufacturer, capture antibody concentrations of 8–10  $\mu\text{g/mL}$  used in conjunction with a detection antibody concentration between 0.5 and 1.0  $\mu\text{g/mL}$  should be able to detect IL-10 samples with concentrations of 10 ng/mL. Hence, our microfluidic platform is able to achieve a LOD consistent with the affinities of these specific antibodies for their antigen.

### a On-Chip Detection of Human IL-10



### b Off-Chip Detection of Human IL-10



**Fig. 3** Chip- and plate-based ELISA detection of human IL-10. a: Average fluorescence signal maxima (for various beads batches and chips) at 605 nm versus human IL-10 concentration. b: Off-chip control dose response curves obtained in 96-well plates. The “speed” ELISA is performed with the same

incubation times as our on-chip assay, whereas the “standard” control is run according to the manufacturer’s instructions. Equations give slopes of linear regions. Arrows point to lower limits of detection. Insets show linear, rather than logarithmic, x-axes. Note that the difference in the scales of the y-axes of the on- and off-chip plots is due to different detectors (on-board vs plate reader, respectively). RFU: relative fluorescence units.

Two types of off-chip ELISAs, which we term “standard” and “speed” ELISAs, were run as controls. The speed ELISA was run to compare the LODs of off- and on-chip ELISAs with the same incubation times. The standard ELISA was run to test whether LODs improved with longer (standard) incubation times. The LOD for the standard assay was 37 ng/mL (Fig. 3b). The off-chip speed ELISA (assay timing control) gave final signals that were five-times lower than the standard ELISA and that were not significantly different than the negative control until 1000 ng/mL (Fig. 3b). Hence, in a plate format, the abbreviated incubation times increase the limit of IL-10 detection by 27-fold, whereas our chip-based platform has a lower LOD than the standard plate assay.

We found that our automated on-chip assay has a greater dynamic range than either of the off-chip control assays. Our assay is linear throughout the entire concentration range and gives a read-out at the highest concentration tested (800 ng/mL) that is 130-times greater than the negative control. We believe that the high surface area of our bead column prevents saturation of the assay.<sup>25,26</sup> In contrast, the standard ELISA is saturated at the highest concentration tested and is only linear up to 333 ng/mL. Between the negative control and 1000 ng/mL, there is only a 10-fold increase in the fluorescence signal. The speed ELISA is linear between 37 and 1000 ng/mL, but its final signal is >5-times lower than the standard control with a minimal increase in fluorescence.

It should be noted that both the capture antibody surface area and the sample volume are greater on our chip than in the plate-based controls (20x and 10x, respectively). We consider the ability to vary the volume of the sample between 10  $\mu$ L and 1 mL to be an advantage of our system, as low abundance analytes can be assayed without a preconcentration step (assuming that sample volume is itself not limiting). Though the beads and plates were both polystyrene, their surface properties were not identical. The beads were unmodified, whereas the plates were treated (by the manufacturer) to make them slightly hydrophilic and better able to bind biomolecules. In the future, using slightly hydrophilic beads could improve capture antibody binding and further improve our limit of detection.

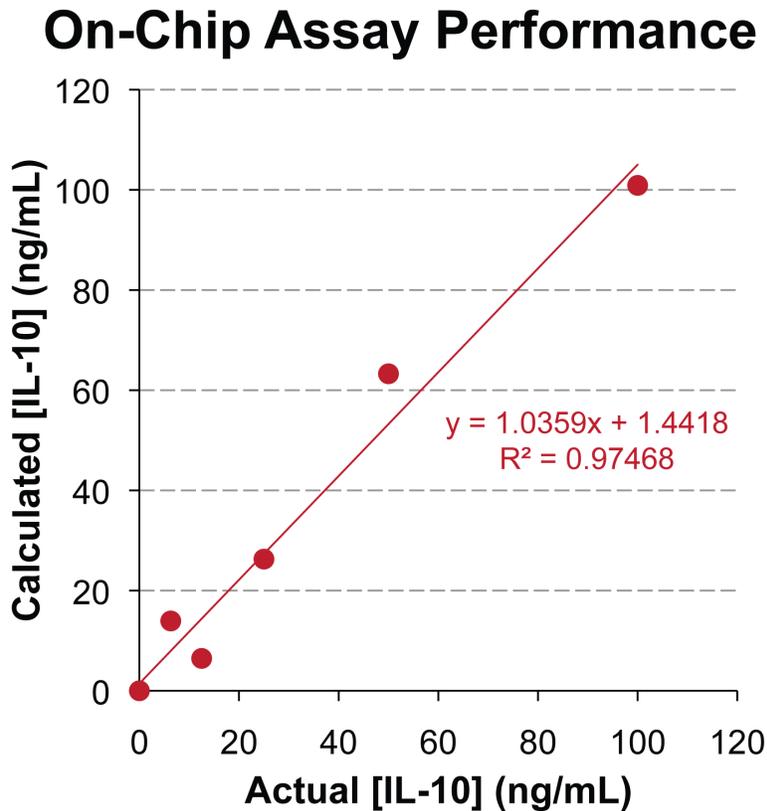
The reproducibility of the LOC system was tested using two different chips and two different bead batches in various combinations. As shown in Table 2, the run-to-run variation was 1–8% whether the same or different beads or chips were used. For comparison, duplicate wells at 333 ng/mL IL-10 run under standard conditions gave ~12% error.

**Table 2.** On-chip data for 400 ng/mL IL-10

Bead Lot	Chip	RFU
1	A	21831
1	A	24320

1	B	23062
2	B	22765

The accuracy of the LOC system at low concentrations was also tested. Human IL-10 was diluted in buffer (between 0 and 100 ng/mL) and run on-chip according to the described protocol. The concentration of IL-10 was then calculated from the dose response curve. When compared, it was found that the calculated concentrations closely matched the actual analyte concentrations, with a slope of 1.03 and an  $R^2$  of 0.97 (Fig. 4).



**Fig. 4** Graph showing the relationship between actual IL-10 concentrations and those calculated from on-chip fluorescence measurements.

## CONCLUSIONS

Our instrument has previously been reported to automate a microfluidic DNA capture and amplification assay.<sup>22</sup> Here we have shown that the same instrument can perform fully automated bead-based ELISAs. The LOD of our on-chip assay surpasses the manufacturer's off-chip plate-based protocol, which requires considerably more hands-on time and takes four times longer (for a single sample). We have designed our automated microfluidic ELISA platform to be versatile. We envision that any off-the-shelf ELISA kit that utilizes a functionalized (*e.g.*, biotinylated) detection antibody, conjugated enzyme, and fluorescent substrate could be easily ported onto our system.

Our valve-free, low-cost chip was originally designed to accept 1-mL dilute samples, but the volume of the sample reservoir could easily be reduced in future versions. However, we feel that the ability of this platform to handle a larger sample input could potentially eliminate the need for pre-analytical sample processing (concentration) otherwise required to detect a given amount of antigen. Likewise, the chip could be multiplexed to run more assays on a sample and to include negative and positive controls. We envision that manufacturing of the disposable, injection-molded chip could involve bead bed and detection antibody loading, while the non-specific assay reagents (enzyme and substrate) could be loaded/stored in bulk on the instrument. A significant advantage of our platform over current automated plate-based ELISAs is that it does not require batching of samples. With further optimization and assay time reduction, our random access platform could potentially reduce results turn-around time and thus provide benefit in a clinical laboratory setting.

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