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# Simultaneous detection of small molecules, proteins and microRNAs using single molecule arrays†

Xu Wang <sup>ab</sup> and David R. Walt <sup>\*ab</sup>

Biological samples such as blood, urine, cerebrospinal fluid and saliva contain a large variety of proteins, nucleic acids, and small molecules. These molecules can serve as potential biomarkers of disease and therefore, it is desirable to simultaneously detect multiple biomarkers in one sample. Current detection techniques suffer from various limitations including low analytical sensitivity and complex sample processing. In this work, we present an ultrasensitive method for simultaneous detection of small molecules, proteins and microRNAs using single molecule arrays (Simoa). Dye-encoded beads modified with specific capture probes were used to quantify each analyte. Multiplex competitive Simoa assays were established for simultaneous detection of cortisol and prostaglandin E2. In addition, competitive and sandwich immunoassays were combined with a direct nucleic acid hybridization assay for simultaneous detection of cortisol, interleukin 6 and microRNA 141. The multi-analyte Simoa assay shows high sensitivity and specificity, which provides a powerful tool for the analysis of many different samples.

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## 1. Introduction

The ability to detect and analyze proteins such as cytokines and toxins, nucleic acids including DNA and RNA, and other analytes such as small-molecule hormones, antibiotics, organic pollutants, pesticide and veterinary drug residues, and mycotoxins, is important for many applications including clinical diagnostics, physiological function research, drug discovery, and environmental and food analysis.<sup>1,2</sup> Current detection methods in clinical and research laboratories rely upon different measurement techniques such as immunoassays, polymerase chain reaction (PCR), and mass spectrometry to detect these analytes.<sup>3,4</sup> Many studies have demonstrated that disease biomarkers, such as small molecules, proteins, DNA and microRNAs, regulate biological processes in a highly interactive manner.<sup>5–7</sup> Thus, simultaneous analysis of multiple biomarkers in a single sample can provide more information for the accurate diagnosis and treatment of diseases.

In recent years, a number of multiplexing technologies have been developed for the detection of multiple small molecules, proteins and nucleic acids.<sup>8,9</sup> The most widely used encoding strategies include fluorescence labeling, spatial separation

using planar arrays, and isobaric labeling for mass spectrometry.<sup>10–12</sup> In contrast to conventional single-plex techniques, multiplexing technologies have rendered the detection of multiple biomarkers more efficient by increasing the amount of biological information obtained from a sample. In addition, a multiplex assay can be performed with smaller sample volumes compared to the detection of each target individually. This feature is particularly important for cases in which sample volumes are limited, such as with cerebrospinal fluid (CSF) or infant blood samples. However, for simultaneous detection of multiple analytes, especially different classes of analytes, it is difficult to establish a universal platform because the detection methods and optimal assay conditions for each analyte are typically different.<sup>13,14</sup> Furthermore, many potentially useful small-molecule, protein or nucleic acid biomarkers in biological samples such as blood, saliva, or other biofluids exist at concentrations well below the detection limits of current methods, necessitating the development of more sensitive technologies.<sup>15,16</sup>

To overcome limitations in analytical sensitivity, our laboratory developed an ultrasensitive digital detection platform based on single molecule arrays (Simoa).<sup>17,18</sup> In Simoa, target analyte molecules are first captured on biofunctionalized paramagnetic beads. A large excess of beads are used relative to the number of target analyte molecules to ensure that there is either zero or one target analyte molecule bound per bead following Poisson statistics. A biotinylated detection probe binds to the captured target analyte molecule, and the formed complex is then labeled with streptavidin- $\beta$ -galactosidase (S $\beta$ G).

<sup>a</sup>Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA. E-mail: dwalt@bwh.harvard.edu; Tel: +1-8573071112

<sup>b</sup>Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

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The enzyme-labeled beads are resuspended in a fluorogenic substrate solution, resorufin- $\beta$ -D-galactopyranoside (RGP), and loaded onto an array of microwells (50 fL) in which each well is able to hold only one bead. The wells are sealed with oil and the fluorescent product generated by the enzymatic reaction is confined within the microwells, ensuring high local fluorescence intensity that can be easily detected by a charge coupled device (CCD) camera.<sup>19,20</sup> The Simoa technique has been previously utilized for ultra-sensitive detection of proteins, DNA and microRNAs, and multiplex assays have been developed for the detection of multiple proteins and microRNAs.<sup>21–25</sup> Moreover, this technique has been used for the detection of small molecules by combining the Simoa assay with a competitive immunoassay, but only single-plex measurements were conducted.<sup>26</sup> In addition, digital immunoassays using droplets,<sup>27–29</sup> femtoliter-sized reactors,<sup>30,31</sup> or tyramide signal amplification<sup>32</sup> have been developed by other groups, and multiplex digital bioassays<sup>33–36</sup> have been established for simultaneous detection of different proteins. However, all these multiplex digital bioassays only detect the same class of analytes, not different classes of analytes.

In this paper, we first describe a multiplex competitive Simoa assay for simultaneous detection of two important small-molecule biomarkers, cortisol and prostaglandin E2 (PGE2). Dye-encoded paramagnetic beads were modified with hapten-BSA conjugates to quantify each analyte. The cross-reactivity of detection reagents was investigated. Next, a 3-plex multi-analyte Simoa assay that incorporates the analysis of small molecules, proteins and microRNAs in one measurement was established by combining competitive and sandwich immunoassays with nucleic acid hybridization. Three widely investigated biomarkers, cortisol, interleukin 6 (IL-6) and microRNA 141 (miR-141) were selected as model targets. The multiplexed measurement of cortisol, IL-6, and miR-141 was validated in saliva samples to demonstrate the practical application of the multi-analyte Simoa assay. To the best of our knowledge, this is the first example of multiplexed detection of proteins, nucleic acids, and small molecules using single molecule measurement methodology.

## 2. Experimental section

### 2.1 Materials

Cortisol solution, 2-(*N*-morpholino)ethanesulfonic acid (MES), Amicon Ultra 0.5 mL centrifugal filters, Tween 20, and bovine serum albumin (A7030) were purchased from Sigma Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), SUPERase-In™ RNase inhibitor (20 U  $\mu$ L<sup>-1</sup>, AM2694), and NHS-PEG4-Biotin were purchased from Thermo Fisher (Waltham, MA). Cortisol-BSA conjugate was purchased from Fitzgerald Industries International (Acton, MA). Prostaglandin E2 (PGE2) was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-PGE2 monoclonal antibody (414013) was purchased from Cayman Chemical (Ann Arbor, MI). Anti-cortisol monoclonal antibody (clone: XM210) was purchased from GeneTex (Irvine, CA). Detection antibodies were biotinylated using NHS-

PEG4-Biotin according to a previously reported method.<sup>37</sup> Synthetic target miR-141 (rUrArArCrArCrUrGrUrCrUrGrUrUrArArArGrArUrGrG) was purchased from IDT. Custom-made locked nucleic acid (LNA) capture (5AmMC12/TTTTTTC + CATCT + TTA + C + C) and detection (+A + GA + CA + GTG + TTA/3Bio/) probes were purchased from Exiqon. Here RNA bases are proceeded by “r” (*i.e.* rA, rC, rG, rU), and LNA bases are proceeded by “+” (*i.e.* +A, +C, +G, +T). Recombinant human IL-6 protein (206 IL), IL-6 capture (MAB206) and detection (BAF206) antibodies were purchased from R&D Systems (Minneapolis, MN). The Simoa HD-X analyzer and Homebrew assay kits were purchased from Quanterix Corporation (Lexington, MA). Homebrew kits include dye-encoded carboxyl-functionalized paramagnetic beads, 50 nM streptavidin- $\beta$ -galactosidase (S $\beta$ G) concentrate, 100  $\mu$ M resorufin  $\beta$ -D-galactopyranoside (RGP), and S $\beta$ G Diluent.

### 2.2 Preparation of capture beads

Dye-encoded carboxylated 2.7  $\mu$ m paramagnetic beads ( $\sim 4 \times 10^8$ ), 488 nm for PGE2 and 647 nm for cortisol, were washed three times with 200  $\mu$ L of bead wash buffer (0.1% Tween 20 in 1 $\times$  PBS, pH 7.4) and twice with 200  $\mu$ L of MES buffer (50 mM MES, pH 6.2). 1 mg mL<sup>-1</sup> EDC in MES buffer was freshly prepared and 200  $\mu$ L was added to the beads and mixed well. The beads were activated on a shaker for 30 min. After activation, the beads were washed once with 200  $\mu$ L of MES buffer. 200  $\mu$ L of 10 mg mL<sup>-1</sup> BSA or 2 mg mL<sup>-1</sup> BSA-cortisol conjugate in MES buffer were added to the activated beads. The beads were incubated at room temperature with shaking for 1 h, and then washed three times with bead wash buffer. The BSA-cortisol conjugate coated beads were stored in 200  $\mu$ L of bead storage buffer (50 mM Tris-HCl with 1% BSA, 1% Triton 100 and 0.15% ProClin 300, pH 7.8) at 4  $^{\circ}$ C for further use.

PGE2 was dissolved in methanol to a final concentration of 1 mg mL<sup>-1</sup>. EDC and sulfo-NHS were reconstituted in MES buffer (50 mM, pH 6.2) to a final concentration of 10 mg mL<sup>-1</sup>. EDC and sulfo-NHS (25  $\mu$ L each) were added to 50  $\mu$ L of PGE2 solution. The mixture was vortexed and incubated at room temperature for 1 h. 30  $\mu$ L of the formed PGE2-NHS ester was added to 200  $\mu$ L of BSA-coated beads in 1 $\times$  PBS. The beads were vortexed, incubated with shaking for 30 min, and then washed five times with bead wash buffer to remove unreacted PGE2-NHS ester. The BSA-PGE2-coated beads were resuspended in 200  $\mu$ L of bead storage buffer and stored at 4  $^{\circ}$ C. In addition, miR-141 capture beads (700 nm) and anti-IL-6 capture beads (488 nm) were prepared according to previously published methods.<sup>23,38</sup> The beads were counted using a Beckman-Coulter multisizer.

### 2.3 Preparation of reagents and assay setup for Simoa assay

A two-step assay configuration was chosen for the multiplexed detection. Specifically, cortisol-BSA-coated beads and PGE2-BSA-coated beads were mixed in the number ratio of 1 : 1. The mixed beads were diluted in sample buffer (5 $\times$  PBS, 1% BSA, 5 mM EDTA, and 0.05% Tween 20) to a concentration of 20 000 beads per  $\mu$ L. Biotinylated detection probes were diluted in



sample buffer to the desired concentrations (90 pM for anti-cortisol antibodies and 6 nM for anti-PGE2 antibodies). SβG concentrate was diluted to 200 pM in SβG Diluent. Cortisol and PGE2 standards were serially diluted to desired concentrations in sample buffer. The reagents including beads, detector, and SβG were placed in plastic bottles (Quanterix). The samples were loaded onto a 96-well plate (Quanterix). All reagents (capture beads, detector antibodies, SβG, enzyme substrate RGP, Wash Buffer 1, Wash Buffer 2, and Simoa Sealing Oil) were purchased from Quanterix and loaded onto the Simoa HD-X Analyzer (Quanterix) based on the manufacturer's instructions. Bead mixture (25 μL) was pipetted into a reaction cuvette. Then, 100 μL of sample and 25 μL of detection antibody mixture were added and incubated for 30 min. The beads were pelleted with a magnet, and the supernatant was removed. Following a series of washes, 100 μL of SβG was added and incubated for 5 min. The beads were washed again, resuspended in RGP solution, and loaded onto the array. The array was then sealed with oil and imaged. Images of the arrays were analyzed, and AEB (average enzyme per bead) values were calculated by the software in the HD-X Analyzer.<sup>39</sup>

For simultaneous detection of cortisol and IL-6, BSA-cortisol-coated beads were mixed with anti-IL6 capture beads (1 : 1 in number). The mixed beads were diluted to a concentration of 20 000 beads per μL. A mixture of detection antibodies, 300 pM for anti-cortisol antibody (XM210) and 6 nM for anti-IL6 detector, was prepared in sample buffer. A mixture of cortisol and IL-6 standards was prepared in sample buffer at desired concentrations. For multiplexed detection of cortisol and miR-141, BSA-cortisol-coated beads were mixed with miR-141 capture beads (1 : 1 in number) and diluted to a concentration of 20 000 beads per μL. A mixture containing 300 pM of anti-cortisol antibody (XM210) and 60 nM of miR-141 detection probe was prepared in sample buffer. A mixture of cortisol and miR-141 standards was prepared in sample buffer at desired concentrations. For simultaneous detection of cortisol, IL-6 and miR-141, BSA-cortisol-coated beads were mixed with anti-IL-6 and miR-141 capture beads (1 : 1 : 1 in number) and diluted to a concentration of 30 000 beads per μL. A mixture containing 300 pM of anti-cortisol antibody (XM210), 6 nM of anti-IL6 detection antibody, and 60 nM of miR-141 detection probe was prepared in sample buffer. A mixture of cortisol, IL-6 and miR-141 standards was prepared in sample buffer at desired concentrations. To make the signal change in the same trend, high concentrations of cortisol were mixed with low concentrations of IL-6 and/or miR-141 to prepare the standards.

For single-plex assay, the capture beads were diluted with sample buffer to a final concentration of 10 000 beads per μL. The analyte standards were serially diluted to desired concentrations in sample buffer. 100 μL of sample, 25 μL of bead mixture, and 25 μL of detection probe were incubated together. All the other steps were the same as those of the multiplex assay.

## 2.4 Saliva samples

Pooled normal human saliva samples were purchased from Innovative Research, Inc. (Novi, MI). Saliva sample was

separated by centrifugation at 13 000g for 20 min, and the supernatant was transferred to a new tube. RNase inhibitor (AM2694) was added to a final concentration of 1 U μL<sup>-1</sup>. Saliva samples were then diluted 50-fold in sample buffer, and different concentrations of cortisol, IL-6 and miR-141 were spiked into the samples for quantitative analysis.

## 2.5 Data analysis

Standard curves were obtained by plotting the signal responses (AEB) against the logarithm of analyte concentrations using Origin software (Origin 9.5). The 4-parameter logistic (equation  $y = A_2 + (A_1 - A_2)/[1 + (x/x_0)^p]$ ) was used for curve fitting in the whole concentration range, where  $A_1$  is the maximum signal,  $A_2$  is the minimum signal,  $p$  is the curve slope at the inflection point, and  $x_0$  is the concentration at the inflection point. For the detection of small molecules,  $x_0$  is the IC50 (analyte concentration causing a 50% inhibition of the maximum response). The lower the IC50, the higher the sensitivity is. For IL-6 and miR-141, the limit of detection (LOD) was calculated as three standard deviations (SDs) above the background. For cortisol, the detection limit was defined at the concentration causing a 10% inhibition of the maximum response. All measurements were performed in triplicate.

# 3. Results and discussion

## 3.1 Principle of multiplex competitive Simoa assay

We developed a multiplex competitive Simoa assay for simultaneous detection of different small molecules. Paramagnetic beads (MBs) were labeled with unique fluorescent dyes to create optically distinct subpopulations of beads that were further modified with specific capture probes to quantify each analyte. Specifically, hapten-BSA conjugates were immobilized to individual subpopulations of beads (Fig. 1). The biofunctionalized MBs were combined and incubated with a sample containing the target small molecules and a mixture of biotinylated detection antibodies specific to the target small molecules. Biofunctionalized MBs and free target small molecules in the sample competitively bind to biotin-labeled antibodies. After incubation and magnetic separation, the supernatant containing unbound antibodies was removed. These beads were then labeled with SβG enzyme *via* biotin-streptavidin interaction. Finally, MBs were loaded into the Simoa disc and imaged to determine: (a) the location and subpopulation identity of individual beads in the femtoliter wells, and (b) the presence or absence of a single enzyme molecule associated with each bead.<sup>24,25</sup> The images were analyzed to determine the average enzyme per bead (AEB) for each bead subpopulation that provides a quantitative parameter for determining the concentration of each analyte.

## 3.2 Multiplexed detection of small molecules

Two widely used small-molecule biomarkers, cortisol and PGE2,<sup>40-42</sup> were selected to test our multiplex assay. Since they are both small molecules (Fig. S1†), the same detection principle (*i.e.* competitive immunoassay) is used. Dye-encoded



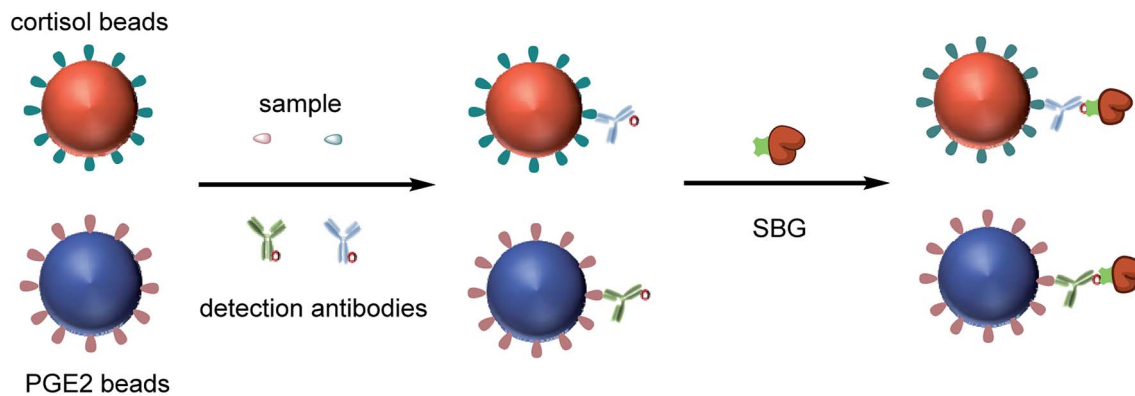


Fig. 1 Schematic illustration of multiplexed detection of two different small molecules using Simoa.

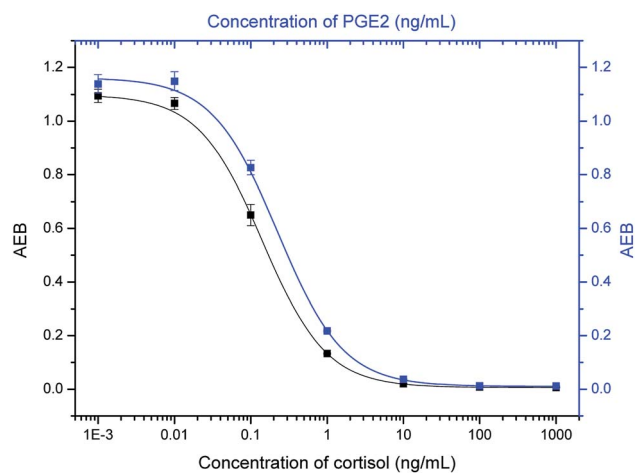


Fig. 2 Response curves of 2-plex Simoa assays for simultaneous detection of cortisol and PGE2.

beads (647 nm) were coated with BSA–cortisol conjugates. For the detection of PGE2, beads (488 nm) were first conjugated with BSA, and then PGE2-NHS ester was added, which specifically reacted with amine groups on BSA molecules (Fig. S2†).

Cortisol and PGE2 were simultaneously detected following the procedures described in the Experimental section. As shown in Fig. 2, with increasing analyte concentration, the signal (AEB) decreased proportionally, because an increase in the number of target molecules leads to a decrease in the number of biotinylated antibody molecules that bind to the MBs, resulting in lower signals. Thus, the signals are inversely proportional to the analyte concentration. The half maximal inhibitory concentration (IC<sub>50</sub>), a parameter that is used to evaluate the sensitivity of a competitive immunoassay, was 0.14 ng mL<sup>-1</sup> for cortisol and 0.22 ng mL<sup>-1</sup> for PGE2, respectively. The sensitivity is comparable with that of single-plex assays, which are 0.12 ng mL<sup>-1</sup> for cortisol and 0.20 ng mL<sup>-1</sup> for PGE2 (Fig. S3†), demonstrating that multiplexing does not compromise sensitivity.

When making multiplexed measurements, the presence of multiple analytes in a sample can potentially introduce cross-reactivity that limits the practical utility of an assay. To assess specificity, PGE2 standard solutions were tested with cortisol–BSA beads and anti-cortisol antibodies, and cortisol standard solutions were tested with PGE2–BSA beads and anti-PGE2 antibodies. As shown in Fig. 3, the signal hardly changes with increasing PGE2 concentrations in the presence of cortisol–BSA beads and anti-cortisol antibodies. Similar results were

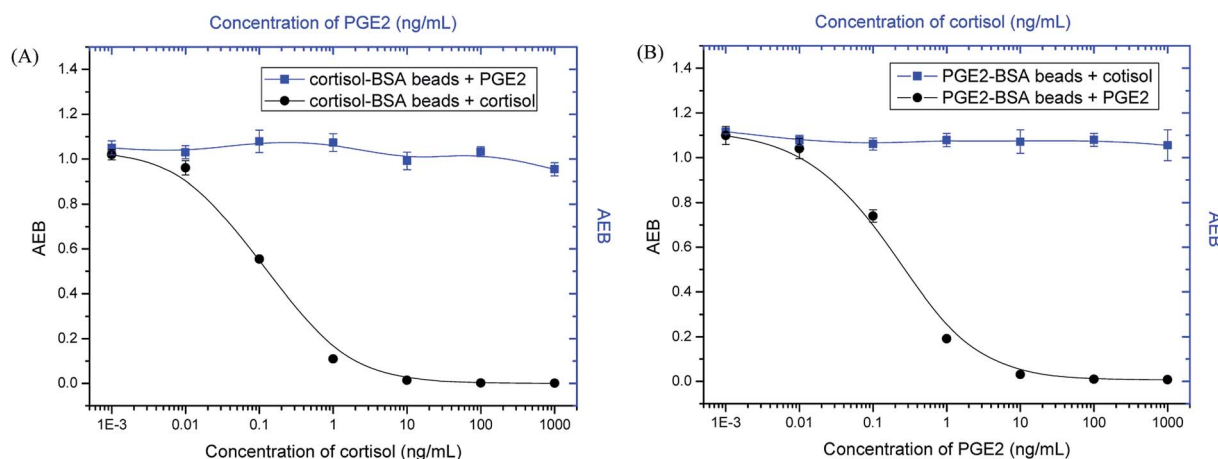


Fig. 3 Detection specificity of the competitive Simoa assays.



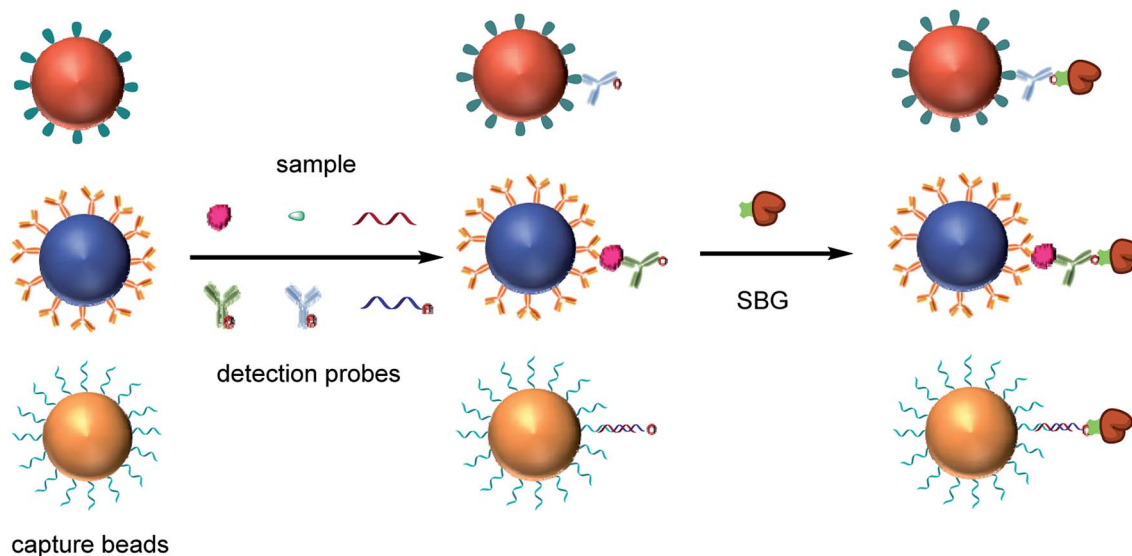


Fig. 4 Schematic illustration of multiplexed detection small molecules, proteins and microRNAs using Simoa.

obtained for the measurements of cortisol when PGE2-BSA beads and anti-PGE2 antibodies were used. In addition, the presence of high concentration of non-target compounds did not interfere with the detection of intended targets (Fig. S4†). These results demonstrate that the assay has high specificity for its intended targets.

### 3.3 Principle of multi-analyte Simoa assay

We also developed a multi-analyte Simoa assay that enables simultaneous detection of small molecules, proteins and nucleic acids (Fig. 4). Hapten-BSA conjugates, protein capture antibodies and target microRNA capture probes were immobilized on three subpopulations of dye-encoded beads, respectively. The biofunctionalized MBs were combined and incubated with a sample containing the target analytes and a mixture of biotinylated detection probes specific to the small

molecule, protein, or microRNA. Hapten-BSA conjugate modified MBs competed with free small molecules in binding to their detection antibodies. For the detection of protein and microRNA, a sandwich complex was formed by capture of the target protein or microRNA on the corresponding capture bead, followed by labeling of the bead with detection probes specific to the protein or microRNA. After incubation and magnetic separation, the beads were washed, labeled with the enzyme  $\beta$ Gal, and detected by enzymatic readout in the Simoa platform.

### 3.4 Simultaneous detection of small molecules, proteins and microRNA

It is challenging to simultaneously detect three different classes of analytes, so we first attempted to detect two analytes at a time. For simultaneous detection of small molecules and

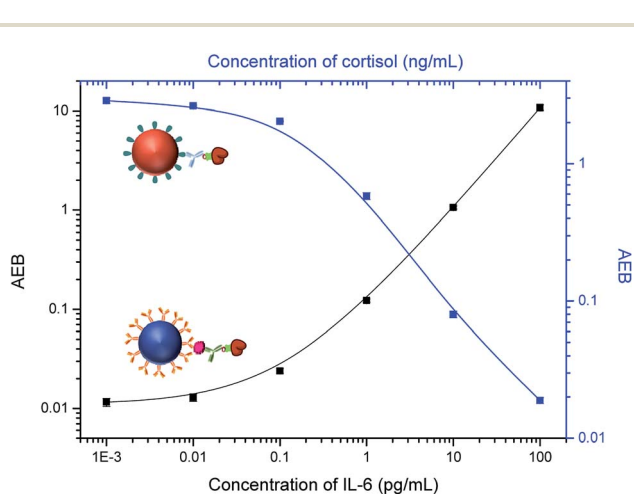


Fig. 5 Response curves of 2-plex Simoa assays for simultaneous detection of cortisol and IL-6.

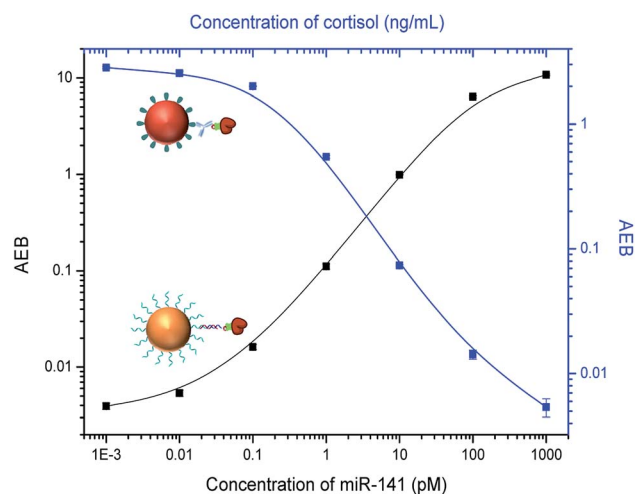


Fig. 6 Response curves of 2-plex Simoa assays for simultaneous detection of cortisol and miR-141.



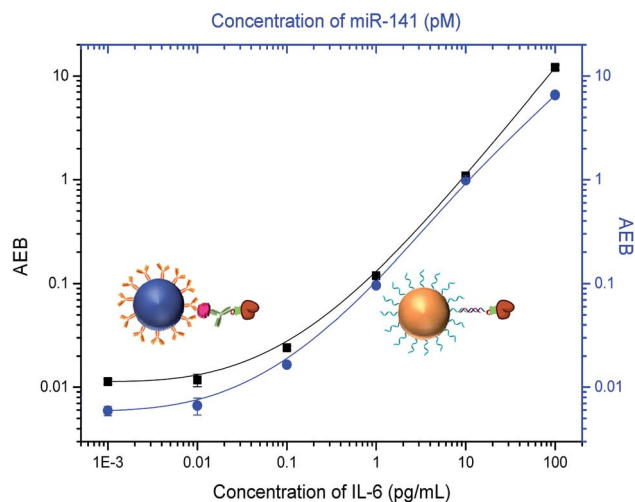


Fig. 7 Response curves of 2-plex Simoa assays for simultaneous detection of IL-6 and miR-141.

proteins, we combined competitive and sandwich immunoassays in one system. IL-6 was selected as a protein biomarker because it is important as both a pro-inflammatory cytokine and an anti-inflammatory myokine.<sup>43,44</sup> Dye-encoded beads (488 nm) modified with anti-IL-6 capture antibodies were mixed with cortisol-BSA beads (647 nm), and then incubated with a sample containing both IL-6 and cortisol and a mixture of anti-cortisol and anti-IL-6 detection antibodies. To stabilize the target proteins and reduce nonspecific binding, a  $1\times$  PBS solution (pH 7.4) containing 1% BSA and 0.05% Tween 20 was used to prepare the samples. As shown in Fig. 5, an increase in the IL-6 concentration correlates to an increase in AEB signal. As expected, an increase in cortisol concentration results in a decrease in AEB signal consistent with the competitive immunoassay format. These results indicate that cortisol and IL-6 can be simultaneously detected in one sample using Simoa.

We next investigated multiplexing of small molecules with microRNAs. Current methods for microRNA detection usually require tedious sample preparation and amplification steps that can bias the results.<sup>23</sup> Here we used a sandwich

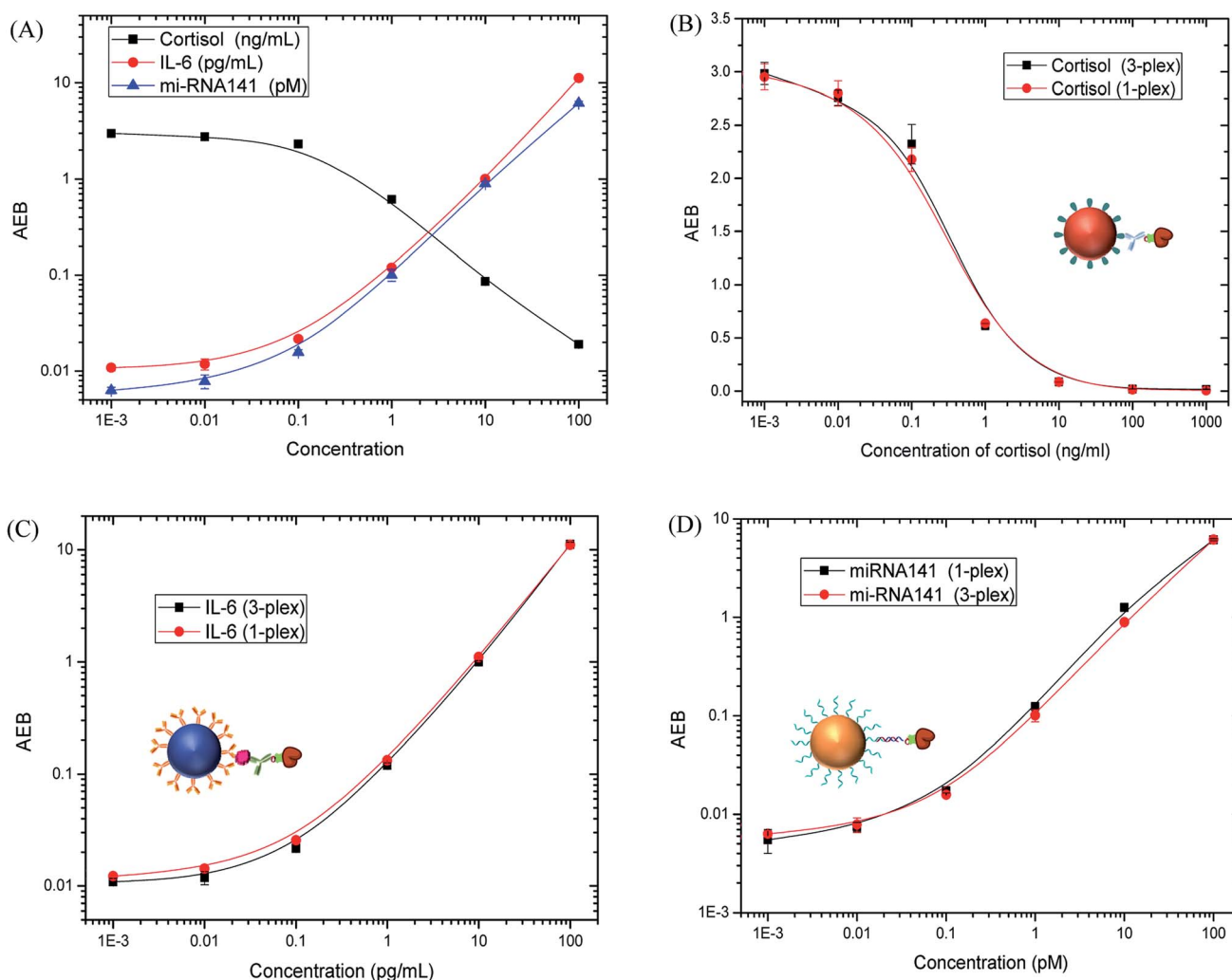


Fig. 8 (A) Response curves of 3-plex Simoa assay for simultaneous detection of cortisol, IL-6 and miR-141, and comparison between 3-plex and single-plex assays for the detection of cortisol (B), IL-6 (C), and miR-141 (D).



Table 1 Multiplexed detection of cortisol, IL-6 and miR-141 in human saliva samples

Spiked	Cortisol		IL-6		miR-141	
	Calculated (ng mL <sup>-1</sup> )	Recovery rate	Calculated (pg mL <sup>-1</sup> )	Recovery rate	Calculated (pM)	Recovery rate
0	0.026	—	0.136	—	0.037	—
0.1	0.125	99.5 ± 0.6%	0.242	106.1 ± 1.4%	0.153	116.4 ± 4.2%
0.5	0.584	111.6 ± 2.3%	0.634	99.5 ± 1.5%	0.472	87.1 ± 2.7%
1.0	1.068	104.2 ± 1.7%	1.075	93.9 ± 1.7%	0.901	86.4 ± 4.6%
5	6.173	122.9 ± 4.6%	4.907	95.4 ± 1.2%	4.789	95.1 ± 2.1%
10	10.374	103.5 ± 5.8%	9.468	93.3 ± 5.2%	9.459	94.2 ± 6.8%

hybridization assay for direct detection of microRNAs without pre-amplification. We chose a widely investigated microRNA biomarker, miR-141,<sup>45,46</sup> as a target. BSA-cortisol beads (647 nm) were mixed with miR-141 capture beads (700 nm) for simultaneous detection of cortisol and miR-141. The target miR-141 molecule was captured by specific nucleic acid probes on the bead surface, which are complementary to the target sequence, and then labeled with a biotinylated detection nucleic acid sequence. Locked nucleic acid (LNA)-modified capture and detection probes were used to increase hybridization specificity. To increase binding affinity of the LNA probes, a high ionic strength buffer, 5 × PBS, was used to prepare the samples. As shown in Fig. 6, the signal increased with increasing miR-141 concentration, while the signal decreased when the concentration of cortisol increased, demonstrating the multiplex assay can be used for simultaneously detecting small molecules and microRNAs.

For simultaneous detection of proteins and microRNAs, a sandwich immunoassay was combined with a direct nucleic acid hybridization assay. IL-6 capture beads (647 nm) were combined with miR-141 capture beads (700 nm) for the Simoa assay. As shown in Fig. 7, the signals for IL-6 and miR-141 both increased with increasing analyte concentrations, demonstrating the Simoa method can be used for the simultaneous detection of IL-6 and miR-141.

After demonstrating the ability to perform two different duplex assays with multiple analyte classes, a 3-plex Simoa assay was established for simultaneous detection of cortisol, IL-6 and miR-141. A competitive immunoassay was combined with a sandwich immunoassay and direct nucleic acid hybridization. The samples were prepared in a buffer solution (5 × PBS, pH 7.4) containing 1% BSA and 0.05% Tween 20, and then measured following the procedures described in the Experimental section. The response curves are shown in Fig. 8A. The LODs for cortisol, IL-6 and miR-141 are 0.031 ng mL<sup>-1</sup>, 0.018 pg mL<sup>-1</sup>, and 0.019 pM, respectively. The detection limits are comparable to those of single-plex assays (0.028 ng mL<sup>-1</sup> for cortisol, 0.022 pg mL<sup>-1</sup> for IL-6 and 0.009 pM for miR-141). In addition, there is negligible difference in the response curves of the 3-plex assay compared to the single-plex assays (Fig. 8B–D), demonstrating that the presence of non-target analytes does not interfere with the detection of target analytes. These results also indicate that the multiplexed format does not compromise the sensitivity and specificity of each Simoa assay for IL-6, cortisol, and miR-141.

### 3.5. Analysis of biological samples

To evaluate the performance of the multi-analyte Simoa assay in complex biological matrices, cortisol, IL-6 and miR-141 standards were spiked into saliva samples and then analyzed to determine recovery. Ribonuclease (RNase) inhibitors were added to saliva samples due to the endogenous presence of RNase in saliva that can interfere with miRNA detection. The concentrations of spiked IL-6, cortisol, and miRNA141 were determined from the calibration curves in Fig. 8A. Recovery rates were calculated by subtracting the measured spiked concentration from the concentration of blank samples and then dividing by the known spiked concentration. As shown in Table 1, satisfactory recovery rates were obtained for cortisol (99.5–122.9%), IL-6 (93.3–106.1%), and miR-141 (86.4–116.4%), indicating the high specificity of the assays. These results demonstrate that the multiplex Simoa assay has the potential to be used for multi-analyte detection in complex samples.

## 4. Conclusions

We developed a multi-analyte Simoa assay for simultaneous detection of small molecules, proteins and microRNAs. Cortisol and PGE2 were simultaneously detected using multiplex competitive Simoa assays. Cortisol, IL-6 and miR-141 in one sample were simultaneously measured by combining competitive and sandwich immunoassays with direct nucleic acid hybridization. We demonstrated that the multiplexed measurement does not compromise the sensitivity and specificity of the individual assays. In addition, we demonstrated the simultaneous detection of cortisol, IL-6 and miR-141 in saliva samples. This method offers an approach to significantly simplify the detection of multiple analytes by combining all assays into a single multiplexed format. This format enables the use of small sample volumes, which is particularly useful for pediatric or precious samples such as cerebrospinal fluid. In addition to multiplexing different analyte classes, the Simoa method is ultrasensitive. Multiplexing combined with ultrasensitivity provides a powerful tool for disease diagnosis. By using different combinations of dye-encoded beads and detection reagents, the established method could be used for simultaneous detection of other analytes in a diverse range of areas, such as clinical diagnostics, environmental monitoring, and food safety.



## Conflicts of interest

The authors declare the following competing financial interest: David R. Walt is a board member and equity holder of Quanterix Corporation. All other authors declare no competing financial interest.

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