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Journal Name

ARTICLE

Single Molecule Array (Simoa) Assay with Optimal Antibody Pairs for Cytokine Detection in Human Serum Samples‡

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Concentrations of cytokines in bodily fluids reflect the physiological or pathological state of the patient and can be used for prognosis, disease diagnosis or for monitoring therapeutic efficacy. However, in the bodily fluids of healthy or sub-healthy individuals, many cytokines are present at concentrations that are near or below the detection limits of current methods. Here we selected antibody pairs to be employed in the single molecule array (Simoa) assay for ten cytokines including GM-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, and IL-10. The limits of detection (LODs) obtained were as low as 90 aM - 6 fM. These assays allow detection of cytokines in healthy human serum samples at levels significantly below the detection limits of conventional ELISA assays. We provide detailed antibody pair information as well as the concentration profiles of ten cytokines in healthy human serum to serve as reference data for further ultrasensitive immunoassay development and future clinical applications.

Introduction

Cytokines are signaling molecules that regulate essentially all functions of the immune system. The concentrations of cytokines in the body change upon infection, inflammation, or other immune response activation. For this reason, cytokines have been considered important biomarkers for a variety of diseases. For example, it has been reported that plasma levels of certain cytokines (TNF-α, IL-6, IL-8) are significantly higher in HIV-infected individuals compared with uninfected individuals.[1] It was also found that the concentrations of some cytokines, particularly IFN-γ, are higher in the serum of patients with active tuberculosis than in serum from healthy donors.[2, 3] Cytokines also play a significant role in prognosis, diagnosis and response to therapy. The relative concentrations of various cytokines and their correlation to diseases potentially enable the design of a fingerprint specific to each disease. For example, a profile of 19 cytokines was generated to non-invasively differentiate between benign and malignant thyroid diseases.[4] It has also been reported that cytokine profiles are distinct for different levels of disease severity in sepsis,[5] and six cytokines including GM-CSF, IL-8, IL-10, IL-12, IL-18 and ICP-1, have already been used in diagnosis.[5, 6]

In their role as signaling molecules, cytokines are released from one cell and subsequently sensed by another cell. The release of cytokines into the extracellular milieu results in their dilution. Many cytokines circulate in healthy individuals at very low levels, most of which are below the limits of detection (LODs) of conventional methods.[7] The detection of these cytokines is usually achieved only in the acute disease state when they reach measurable concentrations, which is often well after clinical symptoms occur. Consequently, a highly sensitive cytokine biomarker assay is required for pre-symptomatic disease diagnosis. Here, we developed Single-Molecule Array (Simoa) based assays with low fM or fg/mL LODs for ten cytokines: GM-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, and IL-10. These assays combine the bead-based ELISA format with a high-density microwell array consisting of femtoliter-sized wells to isolate individual beads containing one or more immunocomplexes and their associated enzyme labels.[8-11]

To perform a Simoa assay, the initial steps of the procedure are largely similar to those of conventional bead-based ELISA. By immobilizing capture antibodies on the surface of paramagnetic beads and incubating sequentially with target molecules, biotinylated detection antibody and streptavidin-labeled enzyme, an immunocomplex is formed. Instead of microplates, the beads are loaded into a microwell array consisting of 50,000 wells (46 fL each well) with dimensions matched to the bead diameter such that only one bead can be loaded per well. The wells are then sealed in the presence of non-fluorescent resorufin-β-D-galactopyranoside (RDG) - an enzyme substrate that is converted into a fluorescent product (resorufin) when it is hydrolyzed in the enzymatic reaction.[10, 12] Since the beads containing an enzyme-labeled immunocomplex are confined into extremely small reaction volumes, the concentration of fluorescent product easily reaches a detectable range even if only one enzyme molecule is present in the well.[10] When the concentration of the target cytokine in the sample is extremely low, according to the

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‡ Electronic Supplementary Information (ESI) available: supplemental figures/tables . See DOI: 10.1039/x0xx00000x

Poisson distribution, only one or zero cytokine molecules will be bound to each bead. Counting the wells that generate signal allows quantification of the cytokine molecules because the ratio between the number of beads that carry an immunocomplex and the total number of beads loaded in the microwells (% active beads) can be directly correlated to the cytokine concentration. Measurements based on counting wells that generate signal and wells that contain a bead but do not generate signal are referred to as a binary or digital readout. For higher concentrations of target, direct quantification (analog analysis) of the fluorescence intensity of active wells is applied to obtain the target molecule concentrations. The average number of enzymes per bead (AEB), which can be determined from both digital and analog analysis, is employed as a consistent unit.[9] Since the precise counting of active beads is essential in the data analysis, any interference from the background noise, particularly due to non-specific interactions between the bead and detection antibody (Det Ab), the capture antibody (Cap Ab) and Det Ab, or the Cap Ab and enzyme, will lead to a deviation from the true results. Meanwhile, if the specific binding affinity between the antibody and antigen is too low, insufficient active beads will be identified in the resulting image, which will greatly affect the digital data analysis. These problems become more serious when the concentration of the target cytokine in the sample is extremely low. In a previous report, this challenge was partially addressed by increasing the number of beads per assay while decreasing the enzyme concentration; this protocol was shown to effectively reduce the background signal arising from non-specific interactions between the capture antibody and labeling enzyme.[13] To further develop an efficient assay that will provide a high response with low background, the selection of recognition reagents (Cap Ab and Det Ab) is critical. In our experience, even antibody pairs that have been previously demonstrated in conventional assays are not necessary appropriate for Simoa assays.

In this study, we tested various capture antibodies paired with various detection antibodies and utilized the best-performing pairs in the Simoa assay. LODs were successfully achieved in the range of 90 aM to 6 fM (1 fg/mL to 0.09 pg/mL): much lower than those of conventional ELISA kits. These validated Simoa assays were then applied to determine cytokine levels in healthy human serum samples.

Materials and methods

Preparation of capture beads

Capture antibodies for ten cytokines (GM-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7 and IL-10) were purchased from commercial venders (details can be found in Table S1). Capture antibodies were reconstituted, divided into aliquots, and stored at -20°C or 4°C as recommended. Tween 20, MES (2-(N-Morpholino)ethanesulfonic acid), hydrochloric acid, Tris-HCl (2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride), BSA (Bovine Serum Albumin) and EDTA (Ethylenediaminetetraacetic acid) were purchased from Sigma-Aldrich. PBS (Phosphate-Buffered Saline) and EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) were purchased from Thermo Fisher Scientific. Carboxyl-modified paramagnetic beads (2.7 μm in diameter) were purchased from Agilent Technologies.

The conjugation of capture antibodies to the paramagnetic beads was performed with some modifications (antibody concentration, EDC concentration, beads concentration and the order of reagent addition) to the previously-reported procedure.[10, 14] In the optimized protocol, 100 μL of carboxyl-modified paramagnetic beads (~2.3 × 10^9 /mL) were transferred to a microtube, and then washed three times with 700 μL of bead wash buffer (1% Tween 20 in PBS) and twice with 700 μL of MES buffer (0.05 M MES, pH 6.2). To perform the washing step, the tube containing beads was placed into a magnetic separator for attracting beads to the tube wall and the supernatant was aspirated. The tube was then removed from the magnet, beads were resuspended in the wash buffer and the procedure was repeated. Beads were then incubated with 100 μl of 1 mg/mL detection antibody for 15 minutes while shaking. 10 mg/mL EDC in MES was freshly prepared and 100 μL was added to the beads and mixed well, followed by the addition of 1 mL MES buffer. This mixture was then incubated for 30 minutes while shaking, washed once with 1200 μL bead wash buffer, and incubated with 1% BSA in PBS for 40 min. Beads were then washed three times with washing buffer and stored in 200 μL bead storage buffer (50 mM Tris-HCl with 1% BSA, 1% Triton 100X and 0.15% ProClin 300, pH 7.8) at 4°C.

Detection antibody biotinylation

Detection antibodies for 10 cytokines (GM-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7 and IL-10) were purchased from the commercial venders (details can be found in Table S1), either in biotinylated form or biotinylated in the laboratory using ChromaLink Biotin reagent (Solulink) according to the manufacturer's procedure. Biotinylated antibodies were reconstituted, divided into aliquots and stored at -20°C or 4°C as recommended.

Microarrays

Microwell array fabrication has been previously described. [15] Briefly, optical fiber bundles (Schott North America) containing 50,000 fibers were polished and etched using 0.025 M HCl (Sigma Aldrich) solution to create wells with 4.5 μm diameter and 3.25 μm depth. The surface of the fibers was modified in ethanol with 3.7% N-Cyclohexylaminopropyltrimethoxysilane (Gelest) in a dry and inert atmosphere. Fibers were stored under nitrogen and used within a week after the surface modification.

Antibody selection and assay development

Although commercial antibody pairs for conventional ELISAs exist, many antibody pairs that are efficient for conventional ELISAs did not perform well for ultrasensitive single molecule assays. We tested capture and detection antibodies from

several companies including R&D Systems, BioLegend and Abcam. Capture antibodies were monoclonal while detection antibodies were either monoclonal or polyclonal antibodies that were purchased in their biotinylated form or biotinylated in the lab, as described in Results and discussion.

Many other factors in the assay protocol, such as buffer composition, incubation times and number of washes, were optimized based on the previous report[10, 13] to provide the best performance. The optimized assay protocol is presented below.

Recombinant proteins GM-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6 IL-7, and IL-10 were purchased from R&D Systems, reconstituted as recommended, divided into aliquots and stored at -20°C. The assays were first performed in 96-well plates. Similar to the previous reports,[10] approximately 300,000 antibody coated paramagnetic beads were mixed with 100 µL cytokine calibration solution prepared in assay buffer I (PBS buffer with Tween 20 and 25% newborn calf serum (Life Technologies)) and incubated for two hours while shaking. Beads were then washed six times with assay wash buffer (5X PBS with Tween 20) using a microplate washer equipped with a magnet (BioTek Instruments, Inc.) and incubated for one hour with 100 µL of 1 nM (0.015 µg/mL) biotinylated detection antibody prepared in assay buffer II (PBS buffer with Tween 20 and 0.5% of newborn calf serum). After six washes, the beads were incubated with 100 µL of 20 pM Streptavidin-β-D-galactosidase (SβG, Quanterix) prepared in assay buffer II with 1 mM $MgCl_2$ (Sigma Aldrich). Beads were then washed twelve times with assay wash buffer and once with 10% sucrose buffer.[13] Finally, beads were resuspended in 15 µL of sucrose buffer, 10 µL of which was loaded into the microwells by spinning for 5 min at 3000 RPM. Microarrays were then swabbed with sucrose buffer and dried under vacuum for about 20 min. The bead-based bulk assays were performed in a similar manner for the first three incubation steps. After the incubation with SβG, the beads were washed six times with assay wash buffer and then incubated with 0.1 mM resorufin-β-D-galactopyranoside (RDG, Life Technologies) for 30 mins. 80 µL of product solution was transferred to a 384-well plate and the fluorescence intensity was recorded by a plate reader (TECAN Infinite M200).

Simoa assay imaging

To remove sucrose and prepare for imaging, microarrays were dipped in 1X PBS, swabbed with water, dipped again in 1X PBS and soaked in 0.1 mM RDG for 30 seconds. Microarrays were then immediately set into the holder of a custom-made epifluorescence microscope[10] that allows automatic and controlled sealing of the microarrays with a silicone gasket. Five sequential fluorescence images were taken 30s apart. Fluorescence images were acquired (577 nm excitation, 620 nm emission) and recorded using a CCD camera.[10] Only wells that showed more than a 20% increase in fluorescence intensity over the five frames were considered to be true positive wells. The final calibration curves of ten cytokines (GM-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5 IL-6, IL-7 and IL-10) were plotted with AEB versus cytokine concentration on a log-log scale, as displayed in Fig. S1. When plotted on a normal scale (inserted plots in Fig. S1), calibration curves displayed highly linear responses (linear fitting coefficient R^2= 0.989-0.999).

Testing human serum samples

Serum samples from 15 healthy individuals were obtained from Bioreclamation (Hicksville, NY). Donors were eight men and seven women of different ages and races. Details about the donors are presented in Table S2. For detection of cytokines in the serum of healthy individuals, serum samples were diluted four times in assay buffer I without newborn calf serum, and 100 µL of this mixture was incubated with beads for capture of target molecules. The assay procedure was identical to the assay calibration procedure described above.

Results and discussion

Antibody selection

In a previous report, the influence of kinetic parameters (on-rate and off-rate) of binding reagents on the Simoa assay efficiency was theoretically discussed.[13] Efficient immunocomplex formation and minimal non-specific interactions are critical for successfully developing single-molecule assays, since high non-specific interactions or low specific binding affinity can affect the counting of true active beads, particularly at lower concentrations. Therefore, in order to develop Simoa assays with high sensitivity, proper selection of matching antibody pairs with high target protein binding affinity and low non-specific binding is especially critical.

Antibodies that have low dissociation constants and that bind to target epitopes well exposed in the native protein are, in general, good candidates for efficient antibodies.[16] However, detailed information is often unavailable for many commercial antibodies, and performance of antibodies in a bulk ELISA assay is not always indicative of Simoa assay performance; thus each potential antibody pair must be tested empirically. In practice, the selection of an appropriate antibody pair involves many considerations. Normally, monoclonal antibodies tend to provide lower backgrounds due to single epitope binding. As a result, they are often used as capture antibodies immobilized on a solid surface (e.g. beads or microplate wells) in ELISA assays. We first selected antibody pairs that were marketed by commercial vendors as having been successfully demonstrated in conventional ELISA assays, and we screened these antibody pairs in bead-based bulk assays. Based on this initial screen, the antibody pairs with better performance were further tested in Simoa assays. We found that the antibody pairs of GM-CSF and IL-6 from R&D Systems performed well in Simoa assays (LODs of 0.09 fM and 0.21 fM for GM-CSF and IL-6, respectively). However, the antibody pairs marketed for IL-4 and IL-10 by R&D Systems exhibited little to no signal in Simoa assays (data not shown). Additional pairs from a second vendor (BioLegend) were then

tested and good results were obtained with LODs of 0.62 fM and 0.5 fM in Simoa assays for IL-4 and IL-10, respectively.

When vendor-recommended antibody pairs resulted in poor performance in Simoa assays, i.e., low signal or high background, then combining the antibodies from different vendors is an alternative approach. For example, the antibody pair for IL-1β (purchased from R&D Systems) performed well in bulk assays with LOD < 0.5 pM (Fig. S2A). However, a much higher background was observed in the Simoa assay (Fig. S2B), as the signal could not be differentiated from the background level until [IL-1β] > 10 fM. Meanwhile, another antibody pair for IL-1β (purchased from BioLegend) demonstrated low background but also showed inferior signal response in Simoa (Fig. S2). Mix-and-match tests were carried out among several antibodies from different vendors and the pair with Cap Ab (clone 8516, R&D Systems) and Det Ab (clone JK1B-2, BioLegend) had the best performance with LOD = 0.30 fM (Fig. S2). The same method was applied to identify optimal antibody pairs for IFN-γ and TNF-α (Table S1, Table 1).

Polyclonal antibodies can recognize multiple epitopes per antigen, which improves their binding efficiency. On the other hand, polyclonal antibodies are more likely than monoclonal antibodies to suffer from batch-to-batch variability or poor reliability.[17] These problems were evident in our Simoa assay development for IFN-γ. We tested biotinylated-antibodies with the same cat number (BAF285, R&D Systems) from different batches (lot number), and observed substantially higher background from the newer batch. The S/N was poor within the measured range (Fig. S3). Therefore, if a monoclonal and a polyclonal antibody performed similarly in the assay, the monoclonal antibody was preferred. This rule was applied to IL-5 antibody selection. When Cap Ab (clone: JES1-39D10, BioLegend) paired with either polyclonal (Abcam) or monoclonal Det Ab (clone: JES1-5A10, BioLegend) provided similarly good results in Simoa assays, the monoclonal was selected due to its higher reliability. For some cytokines, such as for TNF-α, monoclonal antibodies from R&D Systems, BioLegend, and Abcam were all tested and none provided good results. However, when the Cap Ab (clone: 28401) from R&D Systems was paired with the Det Ab (polyclonal) from Abcam, a much better Simoa assay was obtained with LOD = 0.72 fM. Therefore, polyclonal antibodies can be a good choice when monoclonal pairs cannot provide adequate sensitivity.

Table S1 summarizes the details for all the antibody pairs we have tested for those ten cytokines. Note that for IL-2, we purchased the antibody pairs from four vendors and tested 16 combinations; however, only the pair from R&D Systems (clone # 5355 and 5334) displayed a relatively high signal response, which was still worse than other cytokines in terms of LODs (Table 2). In addition, IL-5 also had relatively low sensitivity (LOD = 1.67). The number of commercially available antibodies against IL-5 for ELISA applications is quite limited. Most antibodies were produced from genes with clone # TRFK5, 5A10 or 39D10. Testing different capture/detector combinations of these antibodies did not provide better results. The poor performance of both IL-2 and IL-5 antibody pairs is probably due to the low antibody-antigen binding affinity nature for the two cytokines.

We summarize the complete information (including clone code, vendor and cat #) of all antibody pairs exhibiting the best performance in Simoa assays for 10 cytokines in Table 1. Employing these antibodies, we ran the standard assays and screened serum samples in the following sections.

Table 1 Information for optimal antibody pairs used in Simoa assays for ten cytokines. (R&D = R&D Systems; BL = BioLegend)

Cytokine	Cap Ab Clone (vendor, Cat #)	Det Ab Clone (vendor, Cat #)
GM-CSF	6804 (R&D, MAB615)	3209 (R&D, MAB215)
TNF-α	28401 (R&D, MAB610)	Polyclonal (Abcam, ab9635)
IFN-γ	MD-1 (BL, 507501)	25718 (R&D, MAB285)
IL-1β	8516 (R&D, MAB201)	JK1B-2 (BL, 508301)
IL-2	5355 (R&D, MAB602)	5334 (R&D, MAB202)
IL-4	8D4-8 (BL, 500701)	MP4-25D2 (BL, 500803)
IL-5	JES1-39D10 (BL, 500902)	JES1-5A10 (BL, 501006)
IL-6	6708 (R&D, MAB206)	Polyclonal (R&D, BAF206)
IL-7	BVD10-40F6 (BL, 501302)	BVD10-11C10 (BL, 506601)
IL-10	JES3-19F1 (BL, 506801)	JES3-12G8 (BL, 501501)

Simoa assay and LOD

Each assay was conducted using standard solutions prepared in buffer containing 25% newborn calf serum to simulate a human serum sample matrix. By employing the matching antibody pairs with best performance described above in the optimized assay conditions, we successfully achieved LODs for each assay in the low fM range, and the signal response for the lowest measured concentration was clearly distinguishable from the background for most of the assays. The Simoa assay results for GM-CSF, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, and IL-10 are combined in Fig. 1. The individual calibration plots of ten cytokines are displayed in Fig. S1, where calibration curves displayed highly linear responses (linear fitting coefficient R^2= 0.989-0.999). Error bars shown for three replicate measurements indicate good assay precision. Indeed, coefficients of variance (CV) for most of the assays are typically less than 10%.

In Fig. 1, it can be observed that some assays show higher responses than others (for example, compare GM-CSF to IL-2, and IL-1β to IL-10) at the same concentration, even though

Table 2 Limits of detection (LODs) of the various Simoa assays and most sensitive conventional ELISA kits (compared from four companies: R&D Systems, BioLegend, Abcam and BD Biosciences) for ten cytokines. (The LOD was calculated as background response plus two times the standard deviation except the one for IFN-γ from Abcam, which was calculated based on three times its standard deviation).

Cytokine	Simoa assay		Conventional ELISA kits		Enhancement in sensitivity
	fM	pg/mL	pg/mL	Vendor	
GM-CSF	0.09	0.001	0.26	R&D Systems	260
TNF-α	0.72	0.013	0.191	R&D Systems	15
IFN-γ	1.03	0.017	0.69	Abcam	41
IL-1β	0.30	0.0051	0.14	R&D Systems	27
IL-2	5.92	0.089	0.25	R&D Systems	3
IL-4	0.62	0.0093	0.22	R&D Systems	24
IL-5	1.67	0.0217	1.08	R&D Systems	50
IL-6	0.21	0.0043	0.11	R&D Systems	26
IL-7	0.43	0.0073	0.1	R&D Systems	14
IL-10	0.26	0.0048	0.17	R&D Systems	35

they have similar background levels, which is presumably due to the higher binding efficiency between these cytokines and the corresponding antibodies. Also, the background between assays differs from 0.001 to 0.02 AEB, most likely due to different levels of non-specific interactions. Since both binding efficiency and background signal affect the assay sensitivity, as expected, the most sensitive assays are the ones that have both high responses and low backgrounds such as GM-CSF, IL-4, IL-6 and IL-10.

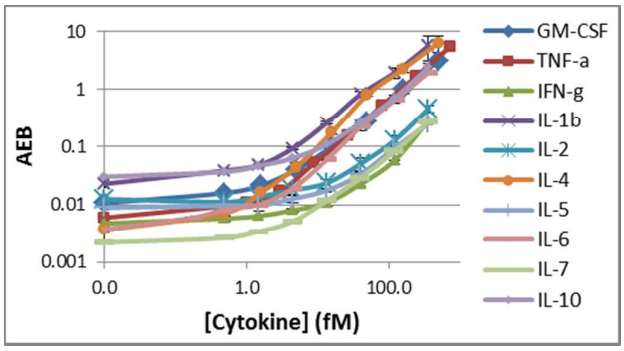

Fig. 1 Simoa assay responses for the different cytokines. Error bars are shown for three replicate measurements.

Table 2 summarizes the LODs of our Simoa assays for ten cytokines, and also displays the lowest LODs of conventional ELISA kits from the four companies (R&D Systems, BioLegend, BD Bioscience, and Abcam), from which we purchased antibodies. It is important to note that the sensitivities of most commercial assays were calculated as background response plus two times its standard deviation, and therefore our results are reported with the same calculation to make a fair comparison. The LODs of our Simoa assays are in the range of 90 aM to 6 fM (1 fg/mL to 0.09 pg/mL) with seven assays having LODs in the sub-fM regime. When compared to conventional ELISA kits, assays demonstrated a 3 to 260-fold improvement in sensitivity over conventional ELISAs.

Real sample analysis

To demonstrate the utility of cytokine detection using our Simoa platform, we employed our assays to detect cytokines in serum samples from 15 healthy donors. Information about the donors is presented in Table S2. Each sample was diluted 4-fold and tested for all ten cytokines. Thanks to the ultrasensitivity of our single-molecule assays, we were able to detect the cytokines GM-CSF, TNF-α, IFN-γ, IL-1β, IL-5, IL-6, IL-7, and IL-10 in all or most of the healthy human samples, most of

Table 3 Median concentrations of eight cytokines in 15 healthy human samples calculated based on detectable samples.

Cytokine		GM-CSF	TNF-α	IFN-g	IL-1b	IL-2	IL-4	IL-5	IL-6	IL-7	IL-10
Median	fM	10.25	73.17	46.72	13.23	34.97	11.92	29.06	46.61	15.73	68.57
	pg/mL	0.14	1.28	0.79	0.22	0.52	0.18	0.41	0.95	0.27	1.28
Detectable/total samples		14/15	15/15	14/15	15/15	3/15	3/15	12/15	15/15	15/15	15/15

which are not detectable by using conventional ELISA kits. However, even with the significant improvement in sensitivity of Simoa assays, IL-2 and IL-4 were still not detectable in most of the serum samples from healthy donors. Concentration profiles and medians for all ten cytokines in healthy human samples are summarized in Fig. 2 and Table 3, respectively. Table 3 also provides the number of detectable samples for each cytokine. Note that the obtained medians were calculated based on the concentration values obtained from detectable samples. The measured cytokine levels for each individual sample (after 4-fold dilution) are also displayed in Fig. S4 in both fM and pg/mL units. When considering the LODs of conventional ELISAs as provided in Table 2, Simoa assays clearly demonstrate superior performance for detection of IL-2, IL-4, IL-1β, GM-CSF, and IFN-γ, most of which are below the LODs of the most sensitive conventional ELISA kits. The #4 and #10 samples have higher levels for several cytokines, which indicate that these donors might have an early infection or some inflammation. However, without any phenotypic information about these individuals other than they were "healthy" when the samples were taken, no conclusions can be drawn.

Conclusions

In summary, we have described the antibody selection process in Simoa assay development, and have provided detailed information for optimal antibody pair selection and usage in our digital ELISA assays, which we believe will benefit those who are interested in developing ultrasensitive immunoassays, especially Simoa assays. The LODs obtained in our Simoa assays are substantially lower than that of conventional immunoassays. By using the developed assays, we were able to measure cytokine levels down to the sub-femtomolar concentration range in healthy human serum samples. The results revealed that normal healthy individuals have detectable levels of GM-CSF, TNF- α, IFN-γ, IL-1β, IL-5, IL-6, IL-7, and IL-10. It is important to note that the range of values for cytokine concentrations varies significantly between individuals. Our ability to quantify these low levels in healthy serum will provide time series baseline measurements to establish the normal ranges within and between individuals. Such time series measurements may ultimately enable these cytokine assays to be used for predicting pre-symptomatic infections.

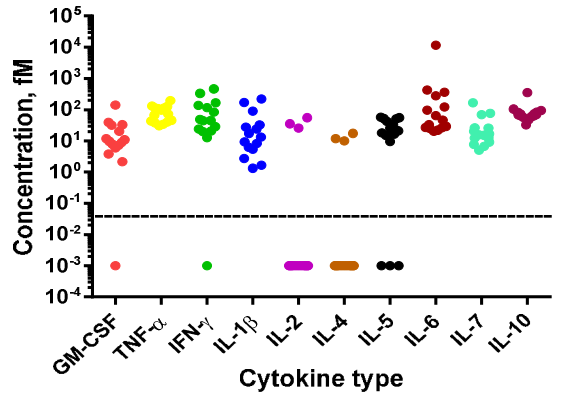

Fig. 2 Cytokine levels in serum samples of healthy individuals determined using the ultrasensitive single molecule assays. The dots under the black dashed line represent measurements below our limits of detection.

Acknowledgements

The authors thank Dr. Shonda Gaylord and Pratyusha Mogalisetti for constructive discussions. This work was supported in part by contract HR0011-12-2-0001 from the Defense Advanced Research Projects Agency.

References

1 J. M. Haissman, L. S. Vestergaard, S. Sembuche, C. Erikstrup, B. Mmbando, S. Mtullu, M. M. Lemnge, J. Gerstoft and H. Ullum, J. Acquir. Immune Defic. Syndr., 2009, 52, 493-497
2 Verbon, Juffermans, D. Van, Speelman, D. Van and P. Van Der, Clin. Exp. Immunol., 1999, 115, 110-113.
3 R. Vankayalapati, B. Wizel, S. E. Weis, P. Klucar, H. Shams, B. Samten and P. F. Barnes, Clin. Infect. Dis., 2003, 36, 24-28.
4 F. Linkov, R. L. Ferris, Z. Yurkovetsky, A. Marrangoni, L. Velikokhatnaya, W. Gooding, B. Nolan, M. Winans, E. R. Siegel, A. Lokshin and B. C. Stack, Proteomics Clin. Appl., 2008, 2, 1575-1585.
5 F. Bozza, J. Salluh, A. Japiassu, M. Soares, E. Assis, R. Gomes, M. Bozza, H. Castro-Faria-Neto and P. Bozza, Crit. Care, 2007, 11, R49.
6 C. Pierrakos and J.-L. Vincent, Crit. Care, 2010, 14, R15.
7 M. A. K. Abrams, M., Genet. Eng. Biotechn., 2013, 33, 28-29.
8 H. H. Gorris, T. M. Blicharz and D. R. Walt, Febs J, 2007, 274, 5462-5470.
9 D. M. Rissin, D. R. Fournier, T. Piech, C. W. Kan, T. G. Campbell, L. Song, L. Chang, A. J. Rivnak, P. P. Patel, G. K. Provuncher, E. P. Ferrell, S. C. Howes, B. A. Pink, K. A. Minnehan, D. H. Wilson and D. C. Duffy, Anal. Chem., 2011, 83, 2279-2285.

10 D. M. Rissin, C. W. Kan, T. G. Campbell, S. C. Howes, D. R. Fournier, L. Song, T. Piech, P. P. Patel, L. Chang, A. J. Rivnak, E. P. Ferrell, J. D. Randall, G. K. Provuncher, D. R. Walt and D. C. Duffy, Nat. Biotech., 2010, 28, 595-599.
11 D. M. Rissin and D. R. Walt, Nano Lett, 2006, 6, 520-523.
12 B. P. English, W. Min, A. M. van Oijen, K. T. Lee, G. Luo, H. Sun, B. J. Cherayil, S. C. Kou and X. S. Xie, Nat. Chem. Biol., 2006, 2, 87-94.
13 L. Chang, D. M. Rissin, D. R. Fournier, T. Piech, P. P. Patel, D. H. Wilson and D. C. Duffy, J Immunol Methods, 2012, 378, 102-115.
14 D. M. Rissin, C. W. Kan, L. N. Song, A. J. Rivnak, M. W. Fishburn, Q. C. Shao, T. Piech, E. P. Ferrell, R. E. Meyer, T. G. Campbell, D. R. Fournier and D. C. Duffy, Lab Chip, 2013, 13, 2902-2911.
15 H. H. Gorris, D. M. Rissin and D. R. Walt, P Natl Acad Sci USA, 2007, 104, 17680-17685.
16 R. Jafari, H. Almqvist, H. Axelsson, M. Ignatushchenko, T. Lundback, P. Nordlund and D. M. Molina, Nat Protoc, 2014, 9, 2100-2122.
17 A comparison between polyclonal and monoclonal, http://www.abcam.com/protocols/a-comparison-between-polyclonal-and-monoclonal, (accessed May 4th, 2015).