Development of a High Sensitivity 10-plex Human Cytokine Assay Using Simoa™ Planar Array Technology

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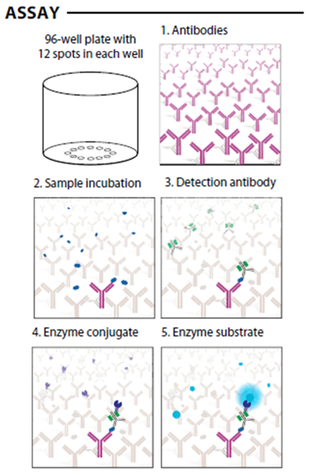
ABSTRACT

Approaches to quantify multiple cytokines have potential to improve understanding of mechanisms and progression of disease, including cancer pathogenesis. However, in healthy humans many cytokines are present at levels below the limits of quantification for established multiplex assays. We developed the Simoa Planar Array to enable protein quantification in biological samples with greater sensitivity than traditional ELISAs. In this study we applied Simoa Planar technology to simultaneous multiplex detection of cytokines, using minimal sample volumes, to achieve sensitivity greater than established multiplex immunoassays. Capture antibodies were printed in a circular pattern in microwells, with biotinylated detector antibodies and chemiluminescent detection used in a sandwich format. Antibody pairs were screened for ability to quantify analytes with minimal cross-reactivity. Pairs targeting IFNγ, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-22 and TNFα were chosen, then assay conditions optimized to minimize matrix interferences. Analytical validation was performed, and circulating cytokine levels measured in normal human serum and plasma. 12.5 µL of human serum/plasma provided sufficient sample volume per replicate. Sub-picomolar LOD was achieved for all analytes; LLOQs ranged from 0.1 – 1.4 pg/ mL. In normal human serum and plasma, > 80% normal samples were quantifiable (above LLOQ) for most analytes. Analytical validation confirmed sensitivity, precision, specificity and dilution linearity. The 10-plex Simoa Planar assay described here combines ultra-sensitivity with powerful multiplexing, enabling simultaneous detection of 10 cytokines in normal biological samples with superior sensitivity .

METHODS



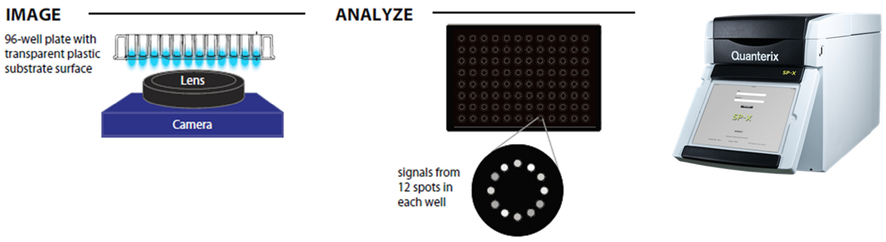


Figure 1 . Assay Workflow

Simoa planar array technology enables high levels of multiplexing via high-precision deposition of multiple capture antibody spots in each well of a microwell plate. Upon analyte binding, biotinylated detector antibodies and chemiluminescent detection reagents used in a ELISA sandwich format generate immunocomplexes which can be imaged by a CCD camera and quantified using image analysis algorithms via the SP-X™ Imaging and Analysis platform. To develop a multiplexed cytokine assay, various combinations of capture antibodies were printed in a circular pattern onto 96-well micro titer plates. Antibody pairs were screened for their ability to quantify analytes while maintaining low background and minimal cross-reactivity. After selection of antibody pairs assay conditions were optimized to minimize matrix interferences and maximize sensitivity. Analytical validation was performed, and circulating cytokine levels measured in normal human serum and plasma. Assay MRD is 4, with 12.5 µL of sample required per data point.

RESULTS

Figure 2. Capture antibody spot layout. Analytes were chosen for significance in immune health and disease and utility in therapeutic development. Capture spot orientation was chosen to minimize risk of signal cross-talk between spots with high levels of bound analyte.

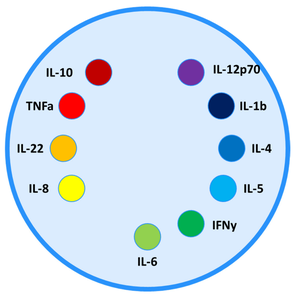
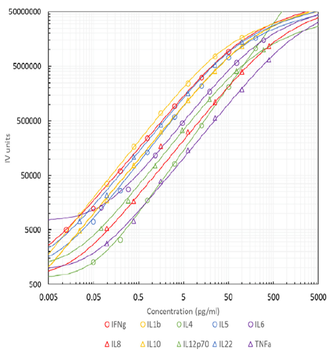


Figure 3: Assay calibration curves. Calibrators were run in triplicate and fitted with a 5 parameter logistic (5PL) regression. Concentration of unknown samples is calculated using the 5PL fit parameters and the IV counts of the unknown samples.



SENSITIVITY	IFNγ	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-22	TNFα
LOD	0.007	0.011	0.046	0.013	0.037	0.115	0.012	0.028	0.01	0.063
LLOQ	0.05	0.1	0.39	0.2	0.59	1.56	0.1	0.29	0.1	0.39
ULOQ	200	400	800	800	1200	1600	400	1200	400	1600

Table 1: Assay Sensitivity and Range. The table above shows the calculated LOD (Lower Limit of Detection), LLOQ (Lower Limit of Quantification) and ULOQ (Upper Limit of Quantification for this assay. LOD is the mean of zero calibration standards + 2.5 standard deviations, LLOQ is the lowest calibration standard with back-calculated concentration pooled CV <20% and relative error <25% multiplied by 4 (MRD), and ULOQ is the highest calibration standard with back-calculated concentration CV <20% and relative error <20%). All values are in pg/mL.

PRECISION	IFNγ	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-22	TNFα
Inter-run CV	10.6%	7.3%	12.1%	6.2%	8.0%	12.2%	10.0%	7.4%	8.8%	11.5%
Intra-run CV	3.9%	4.3%	7.0%	3.7%	3.6%	9.0%	4.7%	4.4%	6.1%	4.8%
Intra-lot CV	7.8%	3.8%	5.0%	4.1%	4.4%	7.3%	6.5%	4.7%	6.1%	7.8%

Table 2: Assay Precision. Three panels (2 serum / 1 plasma) and two controls (low and high concentration calibrators) were diluted 1:4 in Sample Diluent. Mean % CVs based on concentration readings are reported in the table above. The % CV for each individual control and panel was averaged together for Inter-run, Intra-Run and Intra-lot precision. Data shown are derived from a minimum of 13 runs across 3 assay kit lots.

DIL. LINEARITY	IFNγ	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-22	TNFα
Mean	130%	108%	120%	102%	113%	97%	105%	110%	121%	107%

Table 3. Dilution Linearity. Twelve spiked normal human samples (6 serum and 6 plasma) were diluted 4x – 512x. Average recovery over the eight dilutions is shown for each analyte in the 10-plex.

SPIKE RECOVERY		IFNγ	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-22	TNFα
High Spike	Mean	95%	93%	97%	93%	91%	101%	104%	98%	96%	98%
Low Spike	Mean	108%	86%	84%	87%	80%	109%	101%	89%	86%	102%
Average Recovery		101%	90%	91%	90%	85%	105%	102%	93%	91%	100%

Table 4. Spike Recovery. Four normal samples (2 serum and 2 plasma) and a Diluent Control were diluted 1:4 with Sample Diluent and spiked at low and high concentrations with recombinant cytokines. Average percent recovery was based on spiked sample concentration (following subtraction of endogenous cytokine) vs. spiked diluent control concentration.

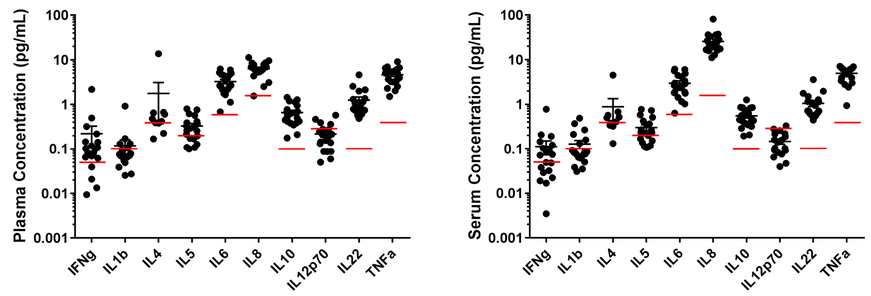


Figure 4. Healthy Human Donor Plasma and Serum Sample Testing. Matched EDTA plasma and serum from 20 healthy donors were tested. Bars depict median with interquartile range red lines indicate LLOQ.

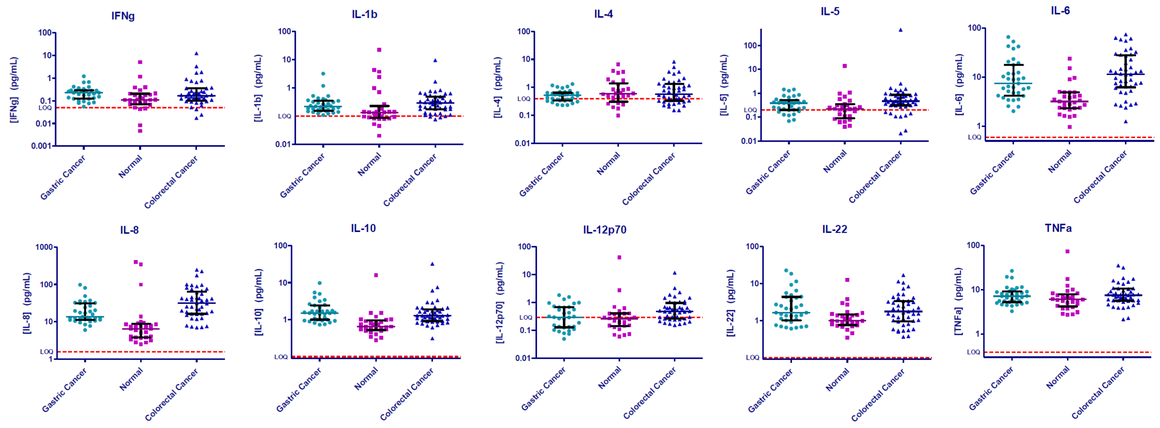


Figure 5. Gastric and Colon Cancer Sample Testing. EDTA plasma from 45 1st line colorectal samples, 35 1st line gastric cancer samples, and 29 apparently healthy normal random samples were measured using the Simoa Corplex™ Human Cytokine 10-plex Panel 1. Data show the three sample populations in relation to the lower limit of quantification (LLOQ). LLOQ cutoffs are as defined by the Simoa CorPlex Human Cytokine 10-plex Panel 1 validation report. Table 5 describes the percentage of samples above the lower limit of quantification (LLOQ).

	IFNγ	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-22	TNFα
Gastric cancer samples % >LLOQ	91.4%	100.0%	62.9%	74.3%	100.0%	100.0%	100.0%	51.4%	100.0%	100.0%
Colorectal cancer samples % >LLOQ	84.4%	91.1%	66.7%	82.2%	100.0%	100.0%	100.0%	73.3%	100.0%	100.0%
Normal samples % >LLOQ	79.3%	62.1%	72.4%	55.2%	100.0%	100.0%	100.0%	34.5%	100.0%	100.0%

Table 5. Percentage of normal, gastric cancer and colon cancer samples reading above LLOQ. EDTA plasma from 45 1st line colorectal samples, 35 1st line gastric cancer samples, and 29 apparently healthy normal random samples were measured using the Simoa Human Cytokine 10-plex assay. Data show the percentage of samples reading above LLOQ for each sample type.

CONCLUSIONS

- We have developed a 10-plex cytokine biomarker immunoassay panel featuring fg/mL sensitivity. This panel comprises IFNγ, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-22 and TNFα.
- Simoa planar array technology combines ultra-sensitivity with powerful multiplexing, enabling simultaneous detection of up to 10 human cytokines in serum and plasma samples.
- Testing of gastric and colon cancer plasma samples indicated that endogenous analytes in the majority of samples tested from these cohorts can be measured above the lower limit of quantification.
- This combination of ultra-sensitivity and cytokine multiplexing provides new opportunities to study the role of low abundance biomarkers in mediating disease pathology and in therapeutic development.

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