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Cytokine Immunoassays with Sub-fg/mL Detection Limits

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1 Abstract

Purpose: Detection limits for high-sensitivity immunoassays are typically in the range of 0.1–1.0 pg/mL, and rarely substantially below 0.1 pg/mL. Typical serum concentrations of many cytokines (e.g., IL-2, IL-4) are well below 0.1 pg/mL and therefore are not measurable in most individuals. Our objective was to develop and characterize an immunoassay format that is 100 to 1000 times more sensitive than the current limits of ELISA technology.

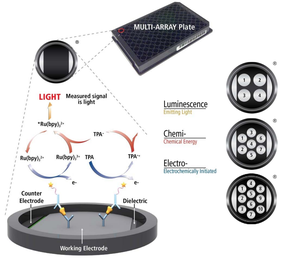
Methods: A next-generation assay format based on MSD's MULTI-ARRAY® electrochemiluminescence technology was developed, requiring only 25 ul of serum or plasma per measurement. The assays were run on the MESO SECTOR ® S 600 and MESO QuickPlex® SQ 120 instruments.

Results: IL-2, IL-4, IL-6, and IL-10 were selected as model analytes. The lower limits of detection achieved for these assays ranged from 0.2 to 0.3 fg/mL, i.e., approximately 1000-fold below the current limits of standard immunoassay performance. The dynamic range was 3 to 4 orders of magnitude, comparable to MSD's standard MULTI-ARRAY assays. Typical intra-plate coefficients of variation ranged between 5% and 15%. Spike recovery and dilution linearity measurements were between 80% and 120%. Approximately 100 serum or plasma samples were evaluated. All 4 cytokines were detectable in all samples with the exception of samples from a single individual whose IL-4 level was undetectable. Serum and plasma concentrations of matched samples correlated. All samples were also measured with a commercial, validated MSD® V-PLEX assay. We found good correlations between the two assay formats for samples that were within the measurable ranges of both formats.

Conclusion: We developed a next-generation assay format that is 100 to 1000 times more sensitive than the current limits of ELISA technology. Detection limits for 4 important cytokines were well below 1 fg/mL. This enables accurate determination of serum concentrations of analytes that were previously undetectable. This new format can be applied to other biomarkers for which current methods are not sufficiently sensitive. The assays can be run on any standard MSD instrument and can be performed within a normal workday using common lab equipment.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates.
We developed the S-PLEX™ assay platform, a next-generation MULTI-ARRAY technology with significantly higher sensitivity.



Electrochemiluminescence Technology

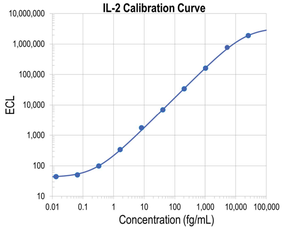
- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

3 S-PLEX Performance Characterization

The performance of 4 cytokine assays was characterized.
Essentially all experiments had the following plate layout:
- Point-symmetrical plate layout; calibrators, QC samples, and unknowns measured in duplicates.
- 7 calibrator levels + zero calibrator; 7x serial dilutions.
- 3 QC samples spanning the assay range and a plasma pool control (QC-4). (1-2 plates were run without QC-4 - BSL-1 experiments).

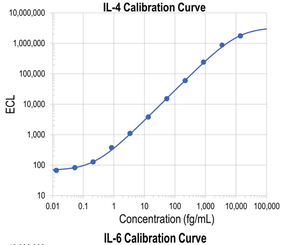
- Performance characterization included determination of limit of detection, upper and lower limit of quantitation, within plate and total reproducibility, spike recovery, and dilution linearity.
- Approximately 80-100 serum or plasma samples were tested, including a set of matched serum, EDTA plasma, and heparin plasma samples from 20 normal individuals, and serum samples from approximately 20 sepsis patients. These samples were also tested on an MSD V-PLEX® panel. In addition, buffy coat stimulated with LPA, PMA, PMS, PWM, or concavalin A was tested.
- All data presented in this poster were generated in an individual assay format, but the S-PLEX technology can also be used in a multiplex format.

4 Calibration Curves



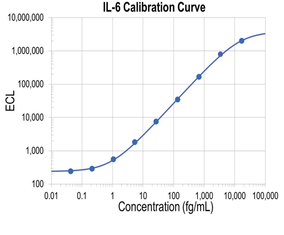
Determination of LLOQ and ULOQ	fg/mL (expected)	ECL counts	fg/mL measured	CV (n=8)	Accuracy
ULOQ	10,500	1,549,667	12,288	6%	117%
	7,000	1,157,333	8,180	14%	117%
	4,620	763,331	4,838	4%	105%
	20	4,556	25	16%	121%
	10	2,170	12	13%	115%
LLOQ	5.1	1,077	5.7	10%	111%
	2.6	526	2.6	7%	102%
	1.3	336	1.5	37%	121%

- IL-2 Assay Range:
  - LOD: 0.2 fg/mL
  - LLOQ: 3 fg/mL
  - ULOQ: 10,500 fg/mL
- A detection limit of 0.2 fg/mL for a 25 µL sample corresponds to approximately 200 IL-2 molecules.



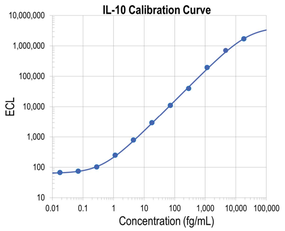
Determination of LLOQ and ULOQ	fg/mL (expected)	ECL counts	fg/mL measured	CV (n=8)	Accuracy
ULOQ	4,000	1,003,389	4,146	9%	104%
	2,667	701,324	2,864	10%	107%
	1,778	481,237	1,950	6%	110%
	9	2,425	10	9%	109%
	4	1,173	4	11%	100%
LLOQ	2.2	593	2.0	18%	91%
	1.1	375	1.1	21%	98%
	0.5	263	0.6	20%	111%

- IL-4 Assay Range:
  - LOD: 0.3 fg/mL
  - LLOQ: 2 fg/mL
  - ULOQ: 4,000 fg/mL
- A detection limit of 0.3 fg/mL for a 25 µLsample corresponds to approximately 300 IL-4 molecules.



Determination of LLOQ and ULOQ	fg/mL (expected)	ECL counts	fg/mL measured	CV (n=8)	Accuracy
ULOQ	10,500	1,692,020	11,599	4%	110%
	7,000	1,532,897	9,534	8%	136%
	4,620	1,096,073	5,419	10%	117%
	7	2,270	7	6%	97%
	3	1,232	3	6%	93%
LLOQ	1.7	775	1.7	18%	98%
	0.9	509	0.8	12%	94%
	0.43	378	0.37	32%	87%

- IL-6 Assay Range:
  - LOD: 0.5 fg/mL
  - LLOQ: 1 fg/mL
  - ULOQ: 5,000 fg/mL
- A detection limit of 0.5 fg/mL for a 25 µL sample corresponds to approximately 500 IL-6 molecules.
- Note: serum and plasma samples should be diluted 10-fold.

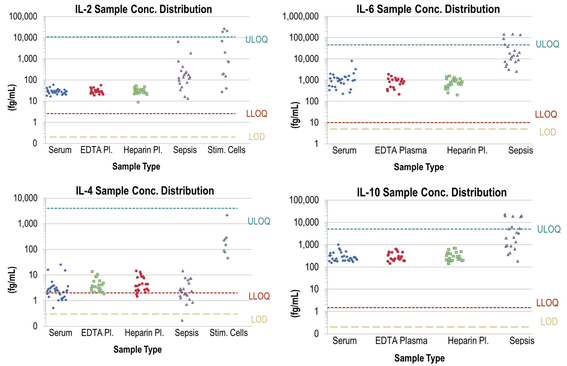


Determination of LLOQ and ULOQ	fg/mL (expected)	ECL counts	fg/mL measured	CV (n=8)	Accuracy
ULOQ	5,333	945,512	5,590	4%	105%
	3,556	661,028	3,840	3%	108%
	2,370	429,470	2,462	4%	104%
	12	2,158	12	5%	102%
	6	1,081	6	11%	98%
LLOQ	2.9	651	3.2	10%	111%
	1.5	375	1.6	16%	111%
	0.7	212	0.7	25%	91%

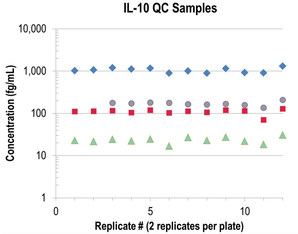
- IL-10 Assay Range:
  - LOD: 0.2 fg/mL
  - LLOQ: 1.5 fg/mL
  - ULOQ: 5,000 fg/mL
- A detection limit of 0.2 fg/mL for a 25 µLsample corresponds to approximately 200 IL-10 molecules.

5 Serum and Plasma Samples

Approximately 80-100 serum or plasma samples were tested, including a set of matched serum, EDTA plasma and heparin plasma samples from 20 normal individuals, and serum samples from 20 sepsis patients. These samples were also tested on an MSD V-PLEX panel. In addition, buffy coat stimulated with LPA, PMA, PMS, PWM, or concavalin A was tested.
IL-2, IL-4, and IL-10 were tested neat (25 ul serum or plasma per test). IL-6 was tested at 10-fold dilution.
All 4 cytokines were detectable in all samples with the exception of samples from a single individual whose IL-4 level was undetectable. For IL-2, IL-6, and IL-10, all the samples were well above the LLOQ and in the middle of the quantitative range. IL-10 could have been diluted 5 to 10-fold.



6 Reproducibility

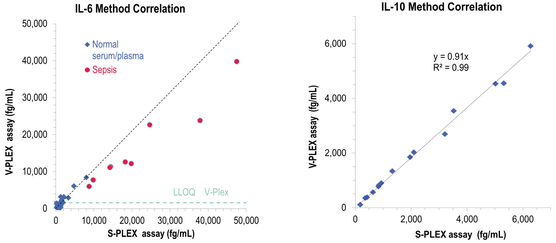


8 plates were run over a period of one month. Each plate included an 8-point calibration curve (duplicates) and two replicates each of 4 QC samples. The plate layout was point-symmetrical, with calibrators in columns 1 and 12, and QC samples in columns 2 and 11. The graph shows total reproducibility data for IL-10.

	Reproducibility (Within-plate)		
	[Conc.]	Mean ECL	CV (n=96)
IL-2	100 fg/mL	17,996	15%
IL-4	100 fg/mL	23,384	5%
IL-6	100 fg/mL	29,431	6%
IL-10	100 fg/mL	19,243	5%

To assess within-plate reproducibility, one 96-well plate was run at a single mid-range calibrator concentration. The table shows within-plate reproducibility.

7 Method Correlation



All samples were also tested on an MSD V-PLEX kit. The two graphs show a method correlation for the IL-6 and IL-10 assays for samples that were within the quantifiable range. For IL-2 and IL-4, most assays were below the LLOQ of the V-PLEX assay.

8 Spike Recovery, Dilution Linearity

Three serum samples, 2-3 EDTA plasma samples and 2-3 heparin plasma samples were spiked with calibrator at three concentrations. Average spike recovery was between 90% and 110% (see Summary Table).
For IL-2, IL-6 and IL-10, three serum samples, 2-3 EDTA plasma samples and 2-3 heparin plasma samples, and for IL-4 eight stimulated buffy coat samples were diluted 2x, 4x and 8x. Average dilution linearity was between 90% and 110% (see Summary Table).

9 Conclusion

A next generation MULTI-ARRAY assay format was developed and characterized. This novel technology is 100 to 1000 times more sensitive than the current limits of ELISA technology. Detection limits for 4 important cytokines were well below 1 fg/mL. This enables accurate determination of serum concentrations of analytes that were previously undetectable.
This new format can be applied to other biomarkers for which current methods are not sufficiently sensitive. The assays can be run on any standard MSD instrument and can be performed within a normal workday using common lab equipment.
The table below summarizes the assay performance for the four cytokine S-PLEX assays.

Summary Table	IL-2	IL-4	IL-6	IL-10
Detection Limit (LOD)	0.2 fg/mL	0.3 fg/mL	0.5 fg/mL	0.2 fg/mL
Lower Limit of Quantitation	3 fg/mL	2 fg/mL	1 fg/mL	1.5 fg/mL
Upper Limit of Quantitation	10,500 fg/mL	4,000 fg/mL	5,000 fg/mL	5,000 fg/mL
Median concentration (serum or plasma, apparently healthy donors)	30 fg/mL	3 fg/mL	900 fg/mL[1]	250 fg/mL
Within-plate CV (n=96)	15%	5%	6%	5%
Spike Recovery (Average; ~9 samples)	97%	109%	93%	106%
Dilution Linearity (Average; ~9 samples)	95%	107%	104%	106%
Sample volume	25 µL	25 µL	2.5 µL	25 µL
Percentage of normal serum/plasma within assay range (~70 samples)	100%	100%	100%	100%

[1]*Note: the IL-6 assay requires 25 µl of 10-fold diluted serum or plasma. Median IL-6 concentrations in 10-fold diluted samples are 90 fg/mL. LOD, LOQ and ULOQ refer to the assay and have not been corrected for dilution of the sample.*

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