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Detection of fg/mL Levels of IL-2, IL-4, IL-6, IL-10, and IL-17A in Serum, Plasma, and Supernatant of Established Cell Models

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1 Abstract

Most immunoassays quantify cytokines with pg/mL sensitivity, which is insufficient to detect those present at low levels. MSD's next-generation S-PLEX℠ assay format was developed to quantify cytokines with fg/mL sensitivity. We described measuring IL-2, IL-4, IL-6, IL-10, and IL-17A levels in normal sera and the supernatants of model cell lines using S-PLEX assays.

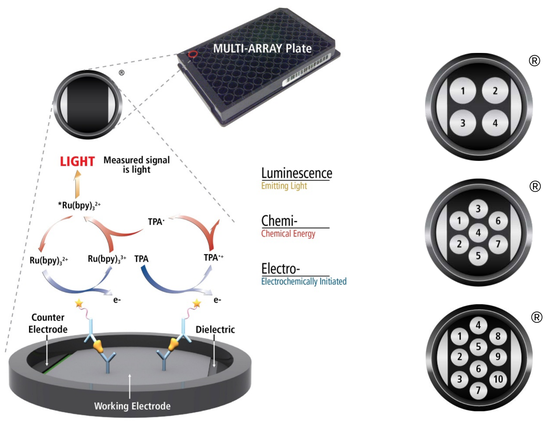
The assays demonstrated a dynamic range of approximately four orders of magnitude. Standard intra-plate coefficients of variation (CVs) ranged from 3%-15% and inter-plate CVs ranged from 8%-18%. The lower limit of detection (LLOD) was <1 fg/mL for all assays except IL-17A (11 fg/mL). IL-2, IL-6, IL-10, and IL-17A were detectable in 100% of normal sera samples (n=36-75) and IL-4 was detectable in 40% of normal sera samples (n=75). The average concentrations were <1 pg/mL for normal samples.

To confirm the sensitivity of these assays and their ability to detect native analytes, we characterized a panel of 23 cell lines that are models for cytokine secretion. As an example, the MOLT-4 cell line derived from acute lymphoblastic leukemia natively expressed detectable levels of IL-2 (1,031 fg/mL), IL-4 (221 fg/mL), IL-10 (60 fg/mL), and IL-17A (30 fg/mL). The expression profiles of these cell lines confirmed the sensitivity of the S-PLEX assays.

The developed S-PLEX cytokine assays provided a 100 to 1,000-fold lower limit of detection (LOD) than standard ELISAs, allowing the determination of baseline cytokine levels in many sample types.

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® microplates. We developed the S-PLEX assay platform, a next-generation MULTI-ARRAY technology with significantly higher sensitivity.

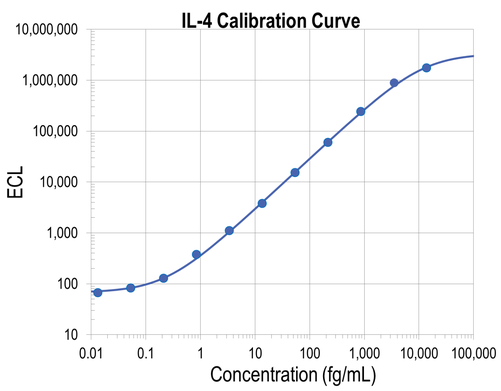


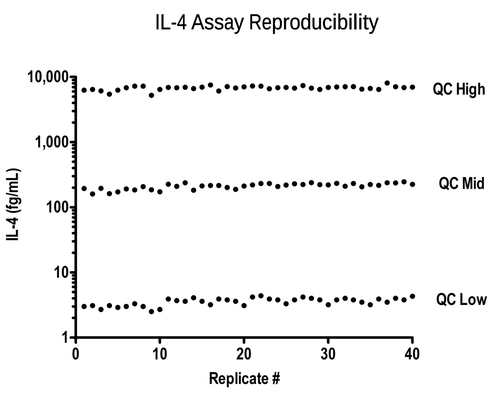
Electrochemiluminescence Technology

- 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 Assay Performance Example (IL-4)

Representative data for the S-PLEX IL-4 assay are shown below. The graph at left shows a representative calibration curve for the S-PLEX IL-4 assay. The LOD was 0.2 fg/mL. Assay reproducibility was assessed by running five replicates of a high, medium, and low QC sample on eight plates (top right) over four days by two operators. Total variability of the IL-4 assay for QC samples with concentrations spanning more than three orders of magnitude was less than 15% (bottom right).





Similar assay performance verification experiments were conducted for the other assays evaluated in this study. The spike recovery and dilution linearity for all analytes were between 80% and 120% (data not shown). Specificity of all assays was also demonstrated by depleting samples with antibodies that were not included in the assay (data not shown).

	% CV			
	Within Run	Between Run	Between Day	Total
QC High	7.0	0.0	3.8	7.9
QC Mid	6.4	1.2	9.5	11.5
QC Low	8.8	0.0	11.4	14.4

4 Assay Range and Native Serum Concentrations (IL-4)

The lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ) were determined by running a series of low (LLOQ-1 to LLOQ-6) and high (ULOQ-1, ULOQ-2) concentrations of calibrator on five plates with four replicates per plate. Each plate included a calibration curve. Total CV (n=20) and average recovery versus expected concentration were calculated for each sample. For this study, LLOQ was defined as the lowest concentration where both total CV was less than 20% and total error was less than 40% (data not shown). ULOQ was defined in an analogous manner as the highest concentration meeting these specifications. The table below (left) shows that the LLOQ for IL-4 was 1.0 fg/mL and the ULOQ was 5,200 fg/mL.

The table on the right shows IL-4 concentrations in apparently healthy serum and plasma samples and in sepsis serum samples.

Sample	Target (fg/mL)	Measured (fg/mL)	%CV (5 plates x 4 replicates)	% Average Recovery
LLOQ1	20	17	14	86
LLOQ2	15	13	11	88
LLOQ3	10	9	10	87
LLOQ4	5.0	4.0	12	79
LLOQ5	3.0	3.0	13	101
LLOQ6	1.0	1.1	14	109
ULOQ1	5,200	4,973	19	96
ULOQ2	4,000	3,583	14	90

	Normal Serum	Normal EDTA Plasma	Normal Heparin Plasma	Sepsis Serum
Median (fg/mL)	2.3	3.7	3.5	2.3
Range (fg/mL)	0.5 - 16	1.5 - 14	0.8 - 14	0.2 - 14
n	30	22	23	20

5 Cytokine Concentrations in Cell Models

Cell models were prepared following published protocols. Cytokine concentrations were determined in neat and 100x diluted cell model supernatants. Cytokine concentrations for some neat samples were above the ULOQ; in those cases the concentrations were determined from the 100x diluted samples. The measured relative abundance of IL-2, IL-4, IL-6, IL-10, and IL-17A in the tested cell systems agreed with literature reports for these model systems.

Sample	Treatment	IL-2 (fg/mL)	IL-4 (fg/mL)	IL-6 (fg/mL)	IL-10 (fg/mL)	IL-17A (fg/mL)
Jurkat E6.1	None	86	67	<1	56	<30
HL60	None	<1.5	2	2	<0.8	41
THP-1	None	<1.5	<0.2	2	3.0	29
THP-1	PMA	<1.5	<0.2	763	5.0	225
BeWo	None	<1.5	<0.2	5,659	<0.8	<30
HDLM-2	None	<1.5	129	842,850	<0.8	<30
HL60	None	<1.5	23	<1	<0.8	<30
K-562	None	11	216	44,969	<0.8	85
MCF7	None	15	<0.2	119	<0.8	<30
MOLT-4	None	1,031	221	<1	60	31
Ku812 Basophil	None	<1.5	18	20,970	<0.8	30
Ku812 Eosinophil	None	<1.5	20	18,320	<0.8	42
H-1650	Protease	<1.5	<0.2	783,350	1.3	<30
H-1650	Trypsin	<1.5	<0.2	663,720	1.5	32
H-1650	LPS + IL-1β	27	<0.2	>1,500,000	<0.8	41
H-1650	LPS + IL-13	10	<0.2	1,455,000	<0.8	30
HCC827	None	<1.5	<0.2	204,960	<0.8	41
H-1650	None	7.9	<0.2	738,200	3.3	<30
Caco-2 apical	None	19	1.2	143	0.9	37
Caco-2	None	42	0.5	230	<0.8	<30
HaCat	None	<1.5	<0.2	4,809	<0.8	34
HaCat	Differentiated	<1.5	<0.2	1,127	<0.8	<30
Ku812	None	<1.5	98	13,330	<0.8	48
PBMCs	None	21,300	48	7,204	2,499	4,061
PBMCs	ConA	>3,000,000	7,339	>1,500,000	270,600	1,785,000
PBMCs	LPS	2,764	46	>1,500,000	1,075,000	4,866
PBMCs	ConA + PMA + Ca ionophore	>3,000,000	14,555	>1,500,000	310,400	2,040,000
PBMCs	LPS + PMA + Ca ionophore	>3,000,000	147,722	>1,500,000	1,750,000	1,577,000

6 Conclusion

We developed a next-generation assay format that is 100 to 1,000 times more sensitive than the current limits of standard ELISA technology. This increased sensitivity enabled accurate quantitation of cytokine levels in serum, plasma, and model systems. The measured relative abundance of IL-2, IL-4, IL-6, IL-10, and IL-17A in the tested cell model systems agreed with the literature and confirmed the validity of these assays.

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