Development and Characterization of an Ultrasensitive Multiplex Biomarker Panel

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PURPOSE

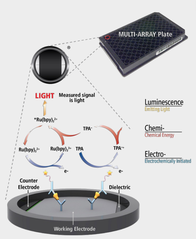
The measurement of circulating biomarkers is an essential aspect of pharmaceutical research and drug development. While a number of biomarkers are readily measurable in the pg/mL to µg/mL range in serum/plasma, many analytes are present at much lower concentrations making them difficult to detect using common immunoassay techniques. Recent advances in immunoassays have enabled measurement of proteins in the fg/mL range, but available assays typically allow detection of only a single analyte at a time and/or require large volumes of clinical samples. To address these challenges, we have developed a high-sensitivity, electrochemiluminescence based multiplex panel sandwich immunoassay platform that enables simultaneous measurement of multiple biomarkers at fg/mL concentrations in a single well — conserving sample and improving workflow efficiency. The multiplex panel uses a single protocol for the simultaneous quantitation of nine analytes: IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, IL-1β, and IL-12p70.

OBJECTIVE

MSD's next generation S-PLEX® platform was developed using electrochemiluminescence technology in order to achieve fg/mL sensitivity to enable multiplexed measurement of low abundant analytes in human samples that previously were not measurable by standard immunoassay methods.

METHOD

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of microplates.



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.
- A carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

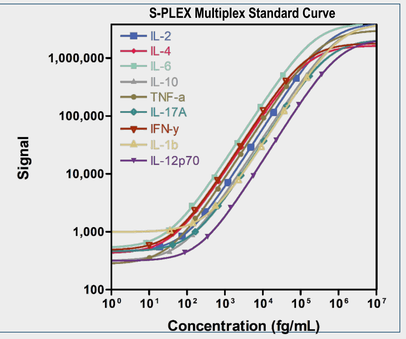
The panel was developed using the S-PLEX platform, which uses an enhanced, electrochemiluminescence reporter technology. Critical components such as immunoassay plates, diluents, blockers, labels, and other reagents were optimized to enable the multiplexing of high-sensitivity assays. Protocols were optimized for ease of use, optimal performance, and robustness. Performance characterization testing of the nine assays in this panel included limit of detection (LOD), lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ), dilution linearity, and spike recovery. Multiple matrices were also interrogated including serum, EDTA/heparin/citrate plasma, CSF, and stimulated cell supernatants.

RESULTS

Calibration Curves, Assay Ranges

The assays were analytically tested using 25 µL sample volumes per well to assess all analytes simultaneously. Of the 9 assays analyzed, 3 had LLODs below 10 fg/mL, 3 had LLODs below 20 fg/mL, and the remaining assays were below 70 fg/mL with dynamic ranges of at least 3 logs and intraplate %CVs <10%. Measurements shown are adjusted to NIBSC/WHO standards.

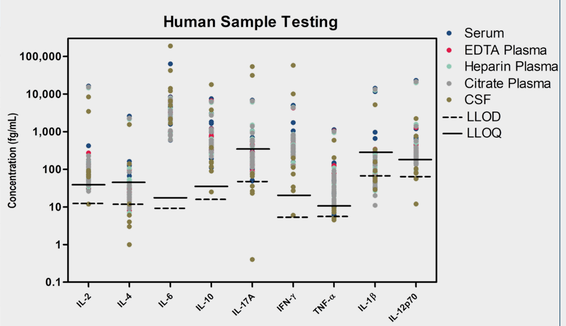
Metric	IL-2	IL-4	IL-6	IL-10	IL-17A	IFN-γ	TNF-α	IL-1β	IL-12p70
Avg. LLOD (fg/ml)	13.0	12.2	9.62	16.0	49.4	5.26	5.74	69.5	67.4
LLOQ (fg/ml)	38.3	43.9	17.0	35.3	340	20.5	10.5	290	180
Assay Range, logs	3.59	3.38	3.37	3.77	3.36	3.71	3.69	3.14	3.55
Hill Slope	1.02	1.02	1.02	1.00	1.02	1.01	1.00	1.01	1.02
Intra-plate %CV	3.2	3.7	4.5	4.2	3.2	3.3	3.9	2.6	6.1



Human Sample Measurement

Endogenous analyte levels were detected in almost all normal serum, plasma, and CSF samples for the majority of assays.

Human Sample Type	Statistic	IL-2	IL-4	IL-6	IL-10	IL-17A	IFN-γ	TNF-α	IL-1β	IL-12p70
Serum (N=37)	Median Conc. (fg/mL)	89	26	3,547	535	191	390	25	71	288
	Range (fg/mL)	30-16,347	ND-542	592-63,522	110-7,567	ND-6,892	128-5,023	6.0-1,135	ND-14,253	142-23,005
	Samples Detected (%)	100	92	100	100	97	100	100	54	100
EDTA Plasma (N=17)	Median Conc. (fg/mL)	111	24	2,173	531	256	350	39	75	303
	Range (fg/mL)	32-15,259	ND-2,210	634-8,021	262-6,583	89-6,475	128-4,246	12-1,043	ND-12,662	179-20,408
	Samples Detected (%)	100	82	100	100	100	100	100	65	100
Heparin Plasma (N=17)	Median Conc. (fg/mL)	92	26	2,357	508	260	374	32	101	317
	Range (fg/mL)	26-14,508	ND-2,148	637-7,882	242-5,996	119-6,097	120-4,469	13-940	ND-12,604	96-19,970
	Samples Detected (%)	100	82	100	100	100	100	100	59	100
Citrate Plasma (N=17)	Median Conc. (fg/mL)	92	19	2,280	502	253	360	26	ND	264
	Range (fg/mL)	27-15,404	ND-2,296	609-7,359	217-6,350	82-6,569	114-4,370	13-1018	ND-13,396	133-21,401
	Samples Detected (%)	100	71	100	100	100	100	100	41	100
CSF (N=8)	Median Conc. (fg/mL)	81	ND	9,292	154	50	94	8.1	75	80
	Range (fg/mL)	ND-8,395	ND-1,556	1,679-189,853	25-17,823	ND-53,791	5.8-57,950	ND-593	ND-5,201	ND-2,236
	Samples Detected (%)	88	25	100	100	50	100	75	50	63
Cell Supernatant (N=4)	Samples Detected (%)	100	75	100	75	75	75	75	100	75



Native Matrix Performance

Dilution linearity and spike recovery results were within the range of 80% to 120% for most assays in plasma and serum, with the exception of IL-2. The average of 4 donors is shown for dilution linearity and the average the of 8 donors is shown for spike recovery.

Matrix	Dilution, X-fold	IL-2	IL-4	IL-6	IL-10	IL-17A	IFN-γ	TNF-α	IL-1β	IL-12p70
Serum	2	80	111	105	101	98	103	102	103	92
	4	73	120	112	119	101	104	104	107	95
	8	71	120	114	121	97	109	102	111	90
EDTA Plasma	2	85	117	114	112	108	117	112	111	91
	4	75	122	119	126	107	118	113	115	96
	8	71	123	117	126	103	119	108	113	88
Heparin Plasma	2	82	109	112	110	104	104	108	103	93
	4	73	113	114	117	100	105	103	106	91
	8	71	124	122	121	105	109	105	105	89
Citrate Plasma	2	80	110	106	111	101	108	103	102	99
	4	72	117	112	121	102	111	105	104	94
	8	71	117	114	127	100	111	105	108	90

Matrix	Spike Level	IL-2	IL-4	IL-6	IL-10	IL-17A	IFN-γ	TNF-α	IL-1β	IL-12p70
Serum	High	158	86	88	102	102	92	109	103	99
	Med	155	88	89	101	99	88	109	103	97
	Low	159	91	91	103	104	93	111	104	99
EDTA Plasma	High	160	83	93	102	94	88	105	97	102
	Med	159	84	90	100	95	85	104	96	96
	Low	161	86	91	102	101	90	109	97	94
Heparin Plasma	High	167	88	95	108	102	103	110	108	101
	Med	159	84	87	98	96	94	104	99	93
	Low	156	86	86	101	97	96	104	97	91
Citrate Plasma	High	162	86	95	102	97	92	107	104	101
	Med	162	88	92	99	97	90	108	104	93
	Low	162	90	92	102	99	94	108	104	95

Assay Cross-Reactivity

Cross-reactivity between the assays was less than 0.5% in all cases.

Detection Ab	Capture Ab: IL-2	IL-4	IL-6	IL-10	IL-17A	IFN-γ	TNF-α	IL-1β	IL-12p70
IL-2		0.04%	0.05%	0.14%	0.07%	0.04%	0.03%	0.06%	0.07%
IL-4	0.29%		0.16%	0.12%	0.17%	0.09%	0.10%	0.21%	0.13%
IL-6	0.05%	0.06%		0.05%	0.03%	0.05%	0.03%	0.15%	0.04%
IL-10	0.45%	0.02%	0.04%		0.04%	0.08%	0.03%	0.03%	0.04%
IL-17A	0.08%	0.03%	0.06%	0.05%		0.04%	0.05%	0.06%	0.29%
IFN-γ	0.02%	-0.03%	-0.02%	0.01%	0.05%		-0.02%	0.00%	0.00%
TNF-α	0.03%	0.02%	0.05%	0.06%	0.06%	0.02%		0.06%	0.01%
IL-1β	0.05%	-0.01%	0.08%	0.02%	0.08%	0.06%	0.01%		0.02%
IL-12p70	0.05%	0.03%	0.06%	0.05%	0.34%	0.04%	0.03%	0.03%	

CONCLUSIONS

We report the development of a highly sensitive, multiplex panel using the MSD® S-PLEX detection platform that combines increased sensitivity in clinically relevant sample types with the ability to measure multiple biomarkers with a single 25 µL sample. Multiple analytes are measured with one experiment, reducing the time needed for experimental setup, shortening assay time to a single workday, and offering improved throughput and sample conservation in comparison with a single analyte detection format. Characterization of this ultra-high sensitivity multiplexed biomarker panel confirmed fg/mL sensitivity, low cross-reactivity, good matrix compatibility, and met acceptance criteria for accuracy and precision. Multiplexing biomarker assays can reduce costs and labor by increasing efficiency, reducing sample volume, and generating more data points per sample in a single run.

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