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Development and Analytical Validation of a Flexible Multiplexing Platform for Cytokine Assays

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1 Abstract

Introduction: Multiplex assays have significant advantages in being able to reduce time and sample volumes; however, multiplexing offers additional challenges, which include developing multiple assays that can be run with the same protocol, reagents, and sample dilution. Furthermore, transferring reagents that work in one assay format to another without compromising assay performance and the integrity of sample measurement is difficult. The U-PLEX® platform enables flexible multiplexing of immunoassays using MSD's MULTI-ARRAY® technology.

Materials and Methods: Antibodies used in the V-PLEX® kits were transferred to the U-PLEX platform by biotinylating the capture antibodies. The assays represented a variety of analyte classes (chemokine, interleukin, interferon), antibody types (monoclonal, polyclonal), and analytical properties (sensitivity, dynamic range, concentration-response slope). During development, antibody concentration, biotin and MSD® SULFO-TAG label ratios, and calibrator concentrations were evaluated and optimized.

Results: Assays were readily transferred to the U-PLEX platform with calibration curves showing signals, sensitivity, precision, and accuracy. Controls for the assays show CVs of <10% within runs. Sensitivities were below 1 pg/mL for many assays. All assays used the same assay diluents. Non-specific binding between assays was shown to be typically <0.1%. We measured 40 human serum and 40 EDTA plasma samples and demonstrated good correlation with V-PLEX assays (r2 > 0.9; slopes 0.8 – 1.2).

Conclusions: The utility and convenience of the U-PLEX platform was demonstrated by easily transferring over 40 human cytokine assays onto the platform without compromising performance. When multiplexed, these assays can be used to make biological measurements on human matrices.

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. The U-PLEX assay platform utilizes 10 unique linkers that specifically bind to individual spots enabling simple and flexible creation of multiplex immunoassays.

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.

Typical U-PLEX Protocol

Create U-PLEX Coating Solution:

- Dilute each biotinylated antibody to 10 µg/mL in coating diluent.
- Add 200 µL of each biotinylated antibody to 300 µL of the assigned linker. Vortex. Incubate at room temperature (RT) for 30 minutes.
- Add 200 µL of Stop Solution. Vortex. Incubate at RT for 30 minutes.
- Combine 600 µL of each U-PLEX-coupled antibody solution into a single tube and vortex. Up to 10 U-PLEX-coupled antibodies can be pooled.

Coat U-PLEX Plate:

- Add 50 µL of multiplex coating solution to each well. Seal plate with an adhesive plate seal and incubate at RT with shaking for 1 hour or overnight.
- Wash plate 3 times with at least 150 µL/well of PBS-T or MSD Wash Buffer.

Complete (Run Assay):

- Add 50 µL of sample, calibrator, or control to each well; shake for 1 hr and wash.
- Add 50 µL of detection antibody solution to each well; shake for 1 hr and wash.
- Add 150 µL of 2X Read Buffer T to each well and read.

3 Calibration Curves and Limits of Detection

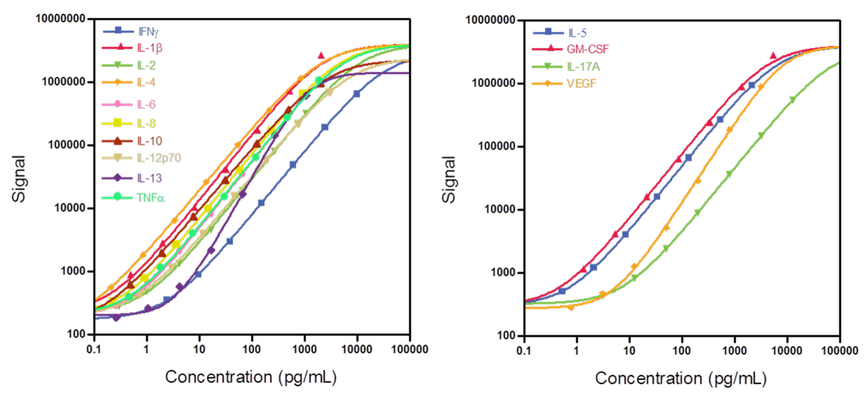
The following table demonstrates the typical data for the first set of 14 biomarker assays tested. Data include Hill slopes and lower limit of detection (LLOD) for the calibration curves. The calibrator is reconstituted and diluted serially (4-fold) to generate a 7-point standard curve. Calibrator 8 in the table indicates the blank.

Top Calibrator Concentration pg/mL														
Assay	IFNγ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNFα	IL-5	GM-CSF	IL-17A	VEGF
Cal 1	9860	2070	1070	868	1080	942	2000	3050	1090	1920	2170	5490	12900	3210

Average Calibrator Signal														
Assay	IFNγ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNFα	IL-5	GM-CSF	IL-17A	VEGF
Cal 1	653938	2667456	320083	1169474	628888	653405	974554	702045	616141	1071801	945567	2803973	549555	901037
Cal 2	194818	737478	81253	365666	156374	172111	363940	220708	142259	278471	270676	898530	150670	188364
Cal 3	49225	175538	19110	102729	34602	41070	107224	59871	17127	64224	66927	246053	36531	28769
Cal 4	12309	41901	4634	26504	8163	9993	28254	15600	2179	15303	16270	64197	8991	5225
Cal 5	3009	10386	1357	6464	2115	2686	7510	3959	578	4066	4033	16212	2409	1273
Cal 6	901	2813	488	1821	663	798	1994	1147	260	1157	1214	4199	840	474
Cal 7	359	902	285	564	285	341	623	441	188	397	511	1160	441	282
Cal 8	173	226	222	179	163	193	189	212	163	171	124	183	91	99

Average LLOD and Hillslope														
Assay	IFNγ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNFα	IL-5	GM-CSF	IL-17A	VEGF
Hillslope	1.02	1.02	1.04	1.01	1.05	1.03	1.00	0.97	1.49	1.06	1.04	1.01	1.02	1.23
LLOD (pg/mL)	1.27	0.11	0.35	0.06	0.21	0.14	0.12	0.35	2.04	1.23	0.26	0.58	2.37	1.88

The following figure demonstrates the typical calibration curves for the 14 biomarkers. The graphs are shown separately for clarity. In practice, any of the markers can be combined to create the desired multiplex.



4 Specificity

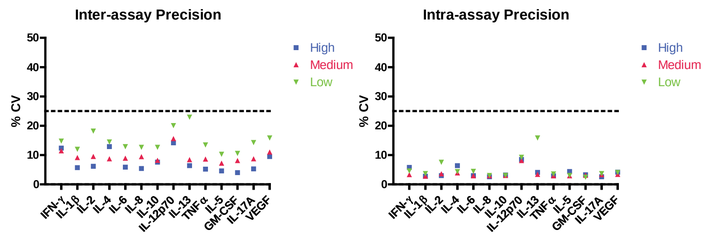
The specificity for each of the markers was evaluated by testing for cross-reactivity for each capture–detector pair with all 40 analytes included in U-PLEX Biomarker Group 1 (human). The following table indicates the analytes tested for specificity.

IFNγ	IL-12p70	Eotaxin	MIP-1β	TNF-β	MIP-3β
IL-1β	IL-13	Eotaxin-3	TARC	G-CSF	SDF-1α
IL-2	TNFα	IP-10	IL-1α	IFN-α2α	Fractalkine
IL-4	IL-5	MCP-1	IL-7	IL-18	CTACK
IL-6	GM-CSF	MCP-4	IL-12p40	TPO	ENA-78
IL-8	IL-17A	MDC	IL-15	I-TAC	IFN-β
IL-10	VEGF	MIP-1α	IL-16	MIP-3α	

- No significant cross-reactivity was observed within the 40 human analytes from the group. Non-specific binding was less than 0.5% for all assays.

5 Precision

Controls were made by spiking calibrator into assay diluent at 3 levels (high, mid, and low) within the quantitative linear range of the assay. The controls were measured using a minimum of 2 replicates tested over 2 days on 9 runs and across 3 U-PLEX platform lots. Average intra-run %CV is the average %CV of the control replicates within an individual run. Inter-run %CV is the variability of controls across multiple runs.

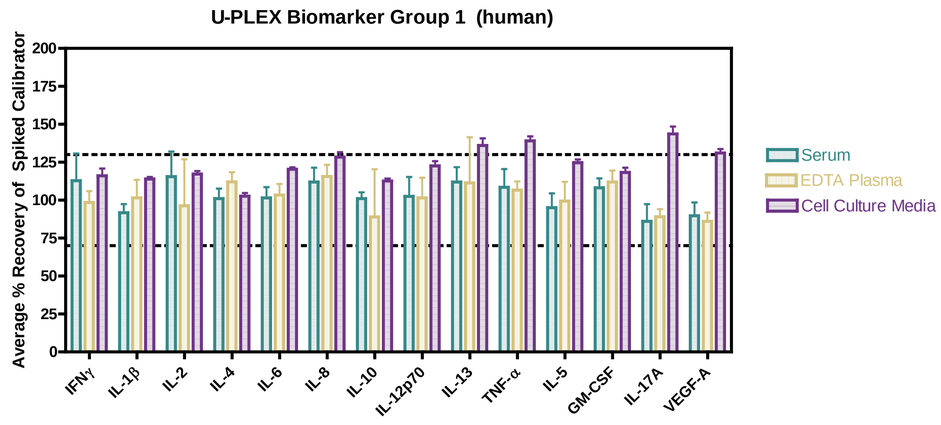


- The %CV (concentration) for both inter- and intra-assay precision were found to be within acceptable limits (≤ 25%), and in most cases the intra-assay CV was less than 10%.

6 Spike & Recovery

Normal human serum (n=5) and EDTA plasma (n=5) samples from a commercial source and cell culture media were spiked with calibrators at 3 levels (high, mid, and low).

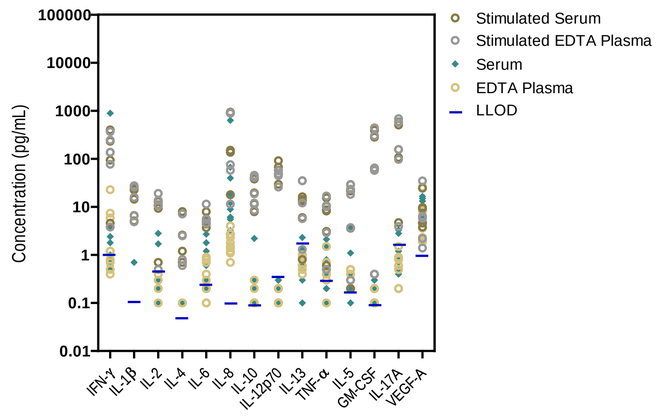
% Recovery = (measured concentration/expected concentration) x 100



- The percent recovery for most of the assays was within the acceptable range (70–130%).

7 Native Sample Testing

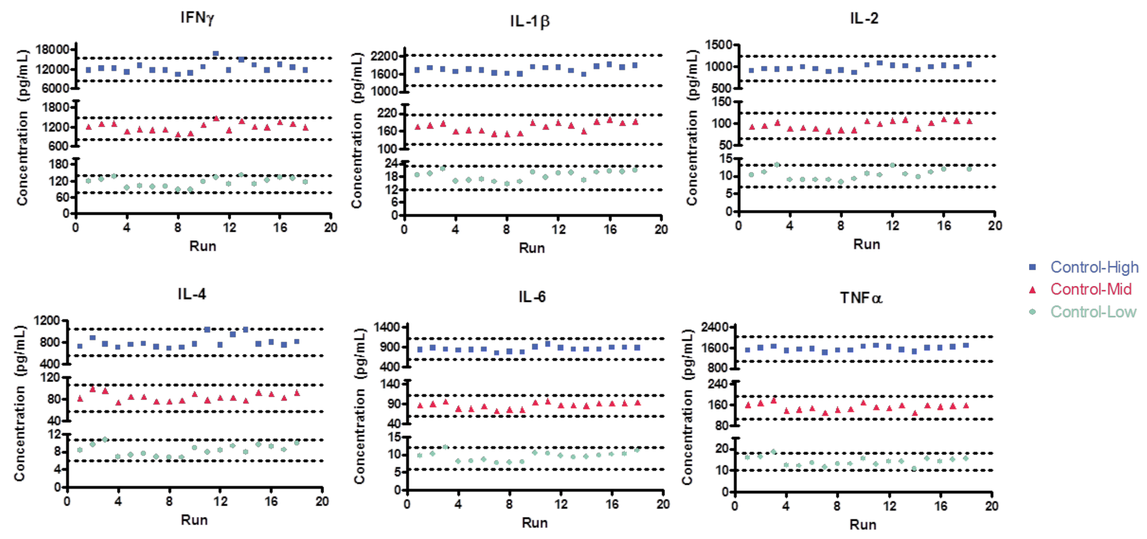
Normal human serum (n=10) and EDTA plasma (n=10) samples from a commercial source were tested neat. To demonstrate the detection of native analyte(s) in matrix, serum (n=5) and plasma (n=5) samples were spiked (spike volume ≤10%) with cell culture supernatants derived from peripheral blood mononuclear cells (PBMCs) that were stimulated with different compounds in vitro. These samples are denoted as stimulated serum and EDTA plasma samples. Results for each sample set are displayed below.



- The native analytes are detectable in normal serum and EDTA plasma as well as in serum and EDTA plasma that were spiked with culture supernatants obtained from stimulated PBMCs.

8 Reproducibility

Reproducibility of assays was evaluated by testing controls at 3 levels across the linear range of the calibration curve. The measured concentrations for 6 representative analytes are plotted in the graphs below. The table shows the average recoveries.

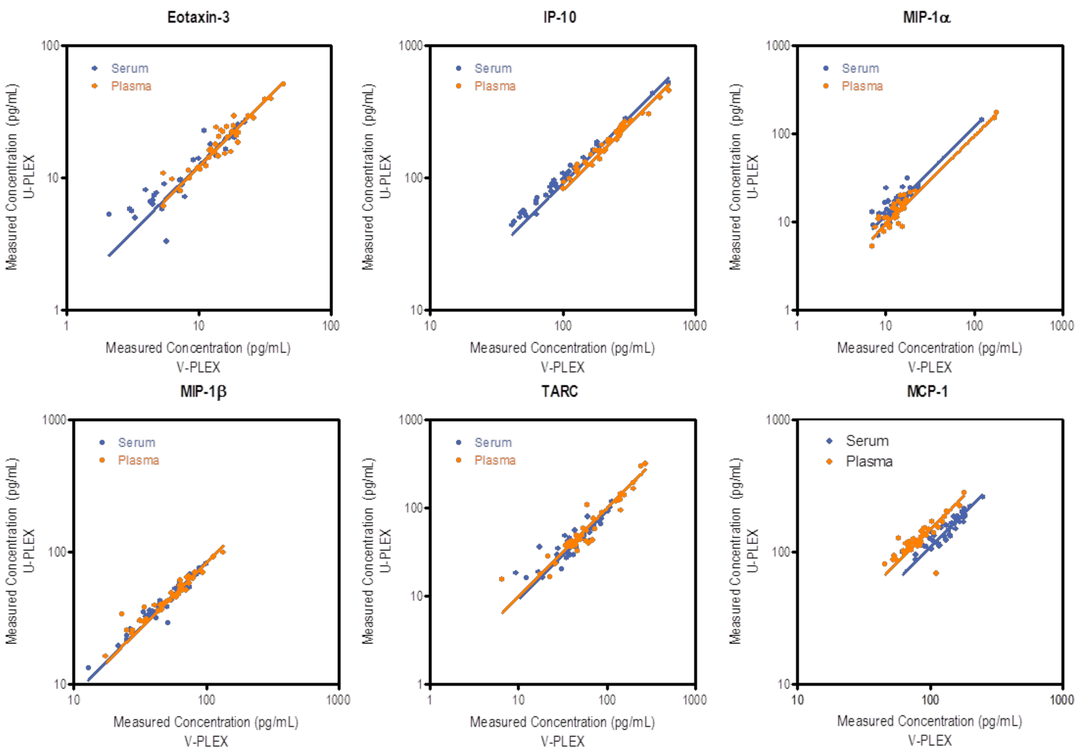


Assay	IFNγ			IL-1β			IL-2			IL-4			IL-6			TNFα		
Level	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3
Average Conc. (pg/mL)	11775	1146	109	1716	166	17	958	95	10	805	82	8	859	86	9	1555	149	14
% Recovery (70-130%)	93	96	96	98	102	103	101	105	110	103	100	99	103	111	116	101	111	113

- The above results indicate that assays developed on the U-PLEX platform are highly reproducible.

9 Comparison with V-PLEX Assays

Forty human serum and 40 EDTA plasma samples were tested with both U-PLEX and V-PLEX assays using the same reagents. The measured concentrations for 6 representative analytes are plotted in the graphs below.



Sample Type	Statistic	Eotaxin-3	MIP-1β	TARC	IP-10	MIP-1α	MCP-1
Serum	r^2 Value	0.98	0.95	0.85	0.99	0.97	0.86
	Slope	0.81	0.83	0.92	0.9	1.19	1.08
Plasma	r^2 Value	0.84	0.95	0.93	0.97	0.99	0.78
	Slope	0.76	0.82	0.99	0.8	0.94	1.47

- Overall, there is excellent correlation between the V-PLEX assays and the same assays performed on the U-PLEX platform.

10 Summary & Conclusion

The U-PLEX technology successfully allows one to design and build multiplex biomarker panels on the sensitive MSD platform rapidly and in a few easy steps:

- Data for the optimization of 14 biomarkers from U-PLEX Biomarker Group 1 (human) are presented.
- Using the U-PLEX reagents, investigators can now combine novel biomarker assays using their own antibodies with assays included in the MSD menu. The antibody sets and calibrators provided in the menu have been optimized for the U-PLEX platform.
- The data generated on the U-PLEX platform correlate well with MSD's validated V-PLEX kits.

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