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

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1 Abstract

Multiplex assays have 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. Moreover, transferring reagents that work in one assay format to another without compromising performance and the integrity of sample measurement is difficult. The U-PLEX® platform enables flexible multiplexing of immunoassays using MSD's MULTI-ARRAY® technology.

Antibodies used in 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.

Assays were readily transferred to the U-PLEX platform with calibration curves showing expected signals, sensitivity, precision, and accuracy. Controls for the assays showed CVs of <10% within runs. Sensitivities were <1 pg/mL for many assays. All assays used the same assay diluents. Non-specific binding between assays was typically <0.1%. We measured 51 human serum and 51 EDTA plasma samples, and demonstrated good correlation with V-PLEX assays (r^2 > 0.9; slopes 0.8 – 1.2).

The utility and convenience of the U-PLEX platform was demonstrated by easily transferring over 50 human cytokine assays onto the platform without compromising performance. When multiplexed, these assays enable multiple measurements from small amounts of human samples.

2 Methods

MSD's electrochemiluminescence (ECL) 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 ten unique linkers that specifically bind to individual spots enabling simple and flexible creation of multiplex immunoassays.

1. COUPLE
Biotinylated Antibody
U-PLEX Linker
U-PLEX Coupled Antibody

2. COAT
U-PLEX Plate with 10 specific linkers

3. COMPLETE
Run personalized combinations of assays

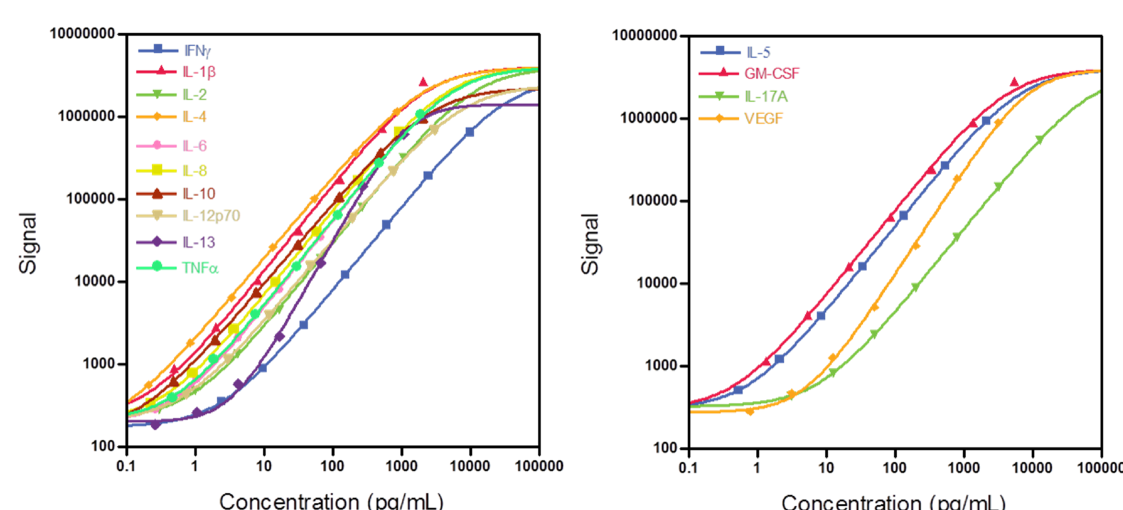
Electrochemiluminescence Technology

- 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 Calibration Curves

The figures at right demonstrate typical calibration curves for 14 biomarkers. Up to ten markers can be combined per plate to create the desired multiplex.

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



Average Hill slope and LLOD														
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

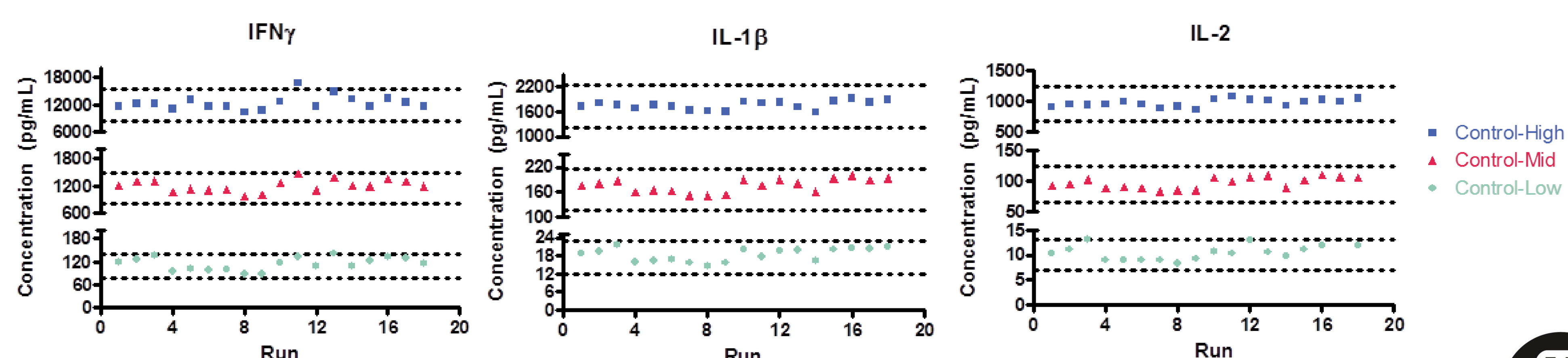
4 Specificity

The specificity for each of the markers was evaluated by testing for cross-reactivity for each capture–detector pair with all 51 analytes included in U-PLEX Biomarker Group 1 (human). No significant cross-reactivity was observed within the 51 human analytes from the group. Non-specific binding was less than 0.5% for all assays.

5 Reproducibility

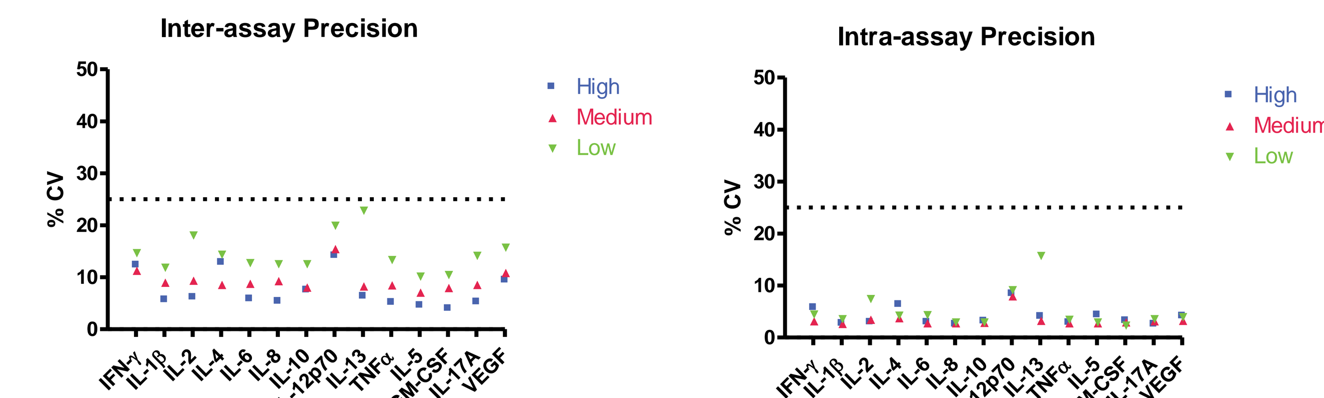
Reproducibility of assays was evaluated by testing controls at three levels across the linear range of the calibration curve. The measured concentrations for six representative analytes are plotted in the graphs below. The table shows the average recoveries. The results indicate that assays developed on the U-PLEX platform are highly reproducible.

Assay	IFNγ			IL-1β			IL-2		
Level	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3	Control 1	Control 2	Control 3
Average Conc. (pg/mL)	11,775	1,146	109	1,716	166	17	958	95	10
% Recovery (70-130%)	93	96	96	98	102	103	101	105	110



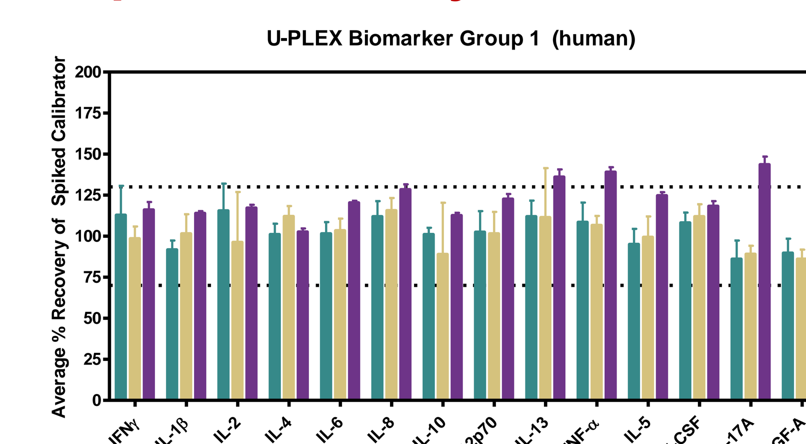
6 Precision

Controls were made by spiking calibrator into assay diluent at three levels (high, mid, and low) within the quantitative linear range of the assay. The controls were measured using a minimum of two replicates tested over two days on nine runs and across three 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%.

7 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 three levels (high, mid, and low).

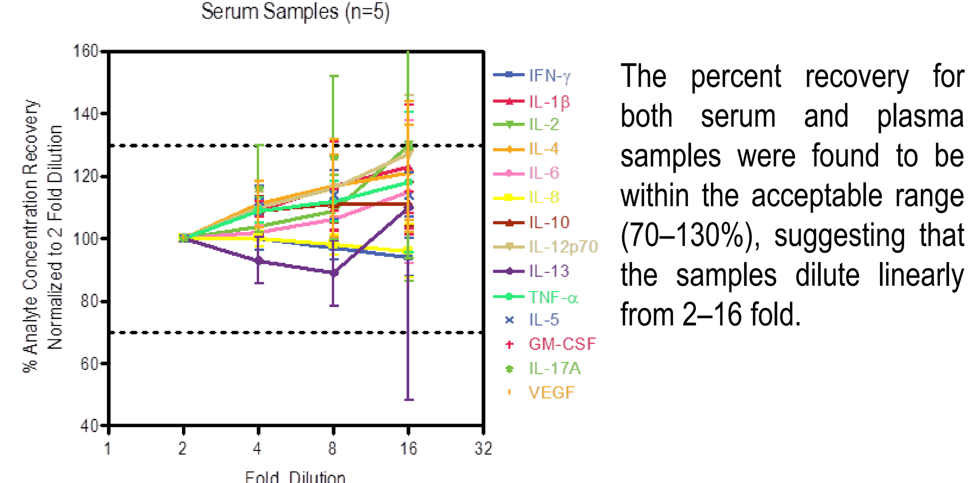
% Recovery = (measured concentration/expected concentration) x 100

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

8 Dilution Linearity

To assess linearity, normal human serum and EDTA plasma samples (both obtained from a commercial source) and cell culture supernatants were spiked with recombinant calibrator and diluted 2, 4, 8, and 16 fold before testing. The average percent recovery shown below is based on samples that measured within the quantitative range of the assay.

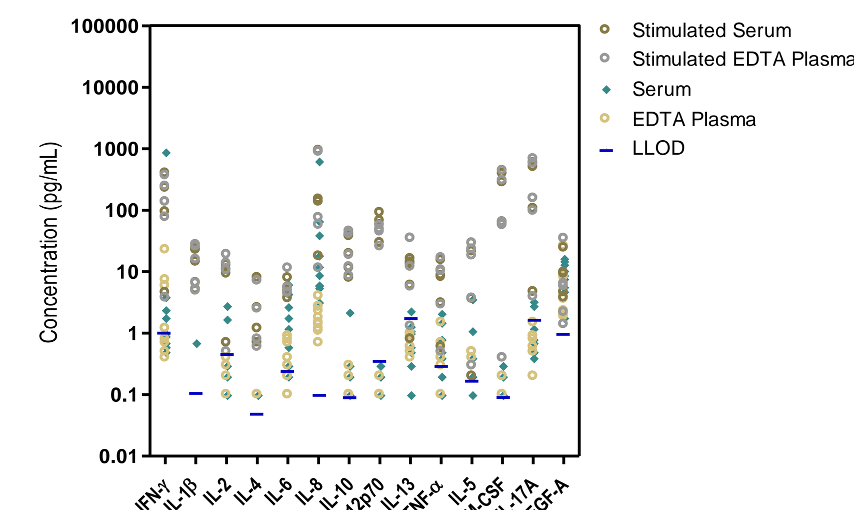
% Recovery = (measured concentration/expected concentration) x 100



The percent recovery for both serum and plasma samples were found to be within the acceptable range (70–130%), suggesting that the samples dilute linearly from 2–16 fold.

9 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.

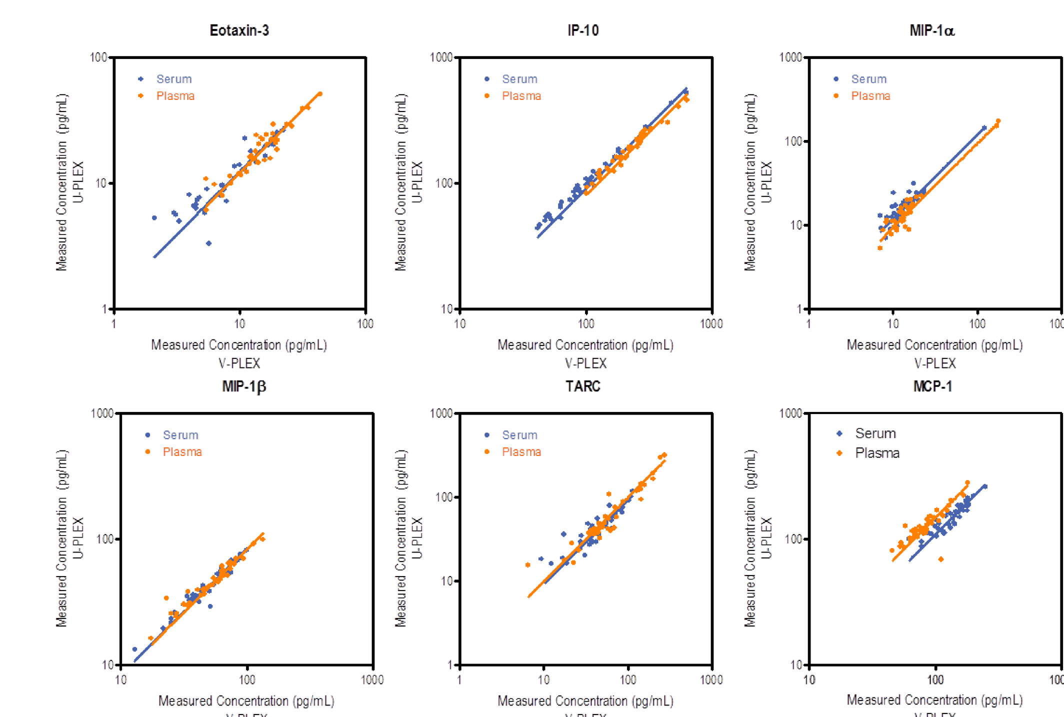


10 Comparison with V-PLEX Assays

Fifty-one human serum and 51 EDTA plasma samples were tested with both U-PLEX and V-PLEX assays using the same reagents. The measured concentrations for six representative analytes are plotted in the graphs at right.

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.



11 Summary and 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 human biomarkers from the 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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