M1028

Rapid Development of Fit-for-Purpose Multiplex Panels on the U-PLEX® Assay Platform

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1 Abstract

Purpose: The goal of this study was to develop more than 20 biomarker assays and assess feasibility of multiplexing. Multiplexing of biomarkers is often a challenge due to differences in analyte abundance in matrix and assay diluent requirements. This leads to the need to reconfigure panels during the early stages of assay development. The U-PLEX assay platform is designed to enable rapid and flexible creation of multiplex immunoassays using MSD's MULTI-ARRAY® technology.

Methods: The U-PLEX platform consists of 10 unique U-PLEX linkers that specifically bind to 10 distinct locations ("spots") at the bottom of each well of a 96-well U-PLEX plate. Each U-PLEX linker has a biotin-binding domain that couples to a biotinylated capture reagent as well as a domain that binds to its corresponding spot on the plate. Antibody pairs were screened on the U-PLEX platform, followed by measurement of analyte levels in serum, plasma, urine, and CSF. The assays were reconfigured into fit-for-purpose assay panels, and then assessed for specificity and matrix tolerance.

Results: Based on the initial assessments of analyte abundance and assay specificity, the assays were configured as several multiplex panels. In the initial assessment, the assay dynamic ranges spanned 3-5 orders of magnitude. However, the samples still required dilutions between 2- and 4000-fold. In addition, improved matrix tolerance was achieved for some assays through use of different assay diluents. Based on these criteria, we were able to group the assays into preliminary multiplex panels in less than one week. Dilution linearity and spike recovery studies demonstrated acceptable matrix tolerance and accurate quantification for most of the assays tested across all matrices (typically between 75-125%). For assays that did not meet these criteria, further assay development or rescreening of antibodies could be performed. The analytes in the final panels typically exhibited non-specific binding of less than 1.0%. Fit-for-purpose panels were established within 3 weeks using as little as 200 µg of each antibody.

Conclusion: The U-PLEX platform facilitated the rapid reconfiguration of fit-for-purpose multiplex panels, reducing assay development time. The resulting panels are suitable for use in early exploratory studies, and are primed for analytical validation.

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:

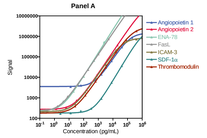
- Add 50 µL of multiplex coating solution to each well. Seal plate with an adhesive plate seal and incubate at RT with shaking for 1 hour or overnight.
- Wash plate 3 times with at least 150 µL/well of PBS-T or MSD® Wash Buffer.

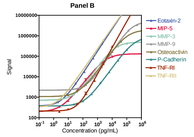
Complete (Run Assay):

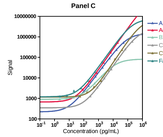
- Add 50 µL of sample, calibrator, or control to each well; shake for 1 hr and wash.
- Add 50 µL of detection antibody solution to each well; shake for 1 hr and wash.
- Add 150 µL of 2X Read Buffer T to each well and read.

3 Antibodies and Reagents Selected

Antibodies were screened and selected (data not shown). The final capture and detection antibodies were conjugated to biotin and SULFO-TAG, respectively. Performance of each assay was confirmed by testing standard curves. A set of 21 assays was grouped into 3 preliminary multiplex panels; reagents and groupings were determined based on abundance levels.
The standard curves shown below and to the right illustrate the sensitivity and dynamic range of the 21 assays in the U-PLEX feasibility study. The graphs display representative data from a single run.





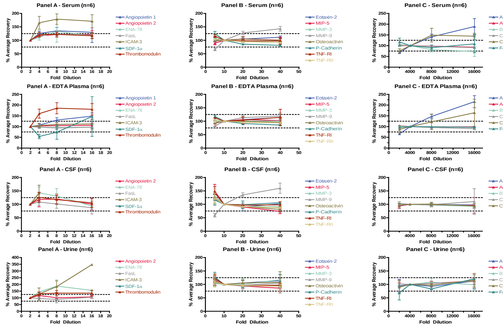


4 Dilution Linearity

To assess dilution linearity, six normal human serum, EDTA plasma, CSF, and urine samples were tested at 4 dilution factors. The initial dilution factor was determined from the literature or measurement of a subset of samples. The selected sample dilution factor was set as 100% recovery. The average % recovery for most of the analytes in the samples fell between 75% and 125%. The % recovery was elevated for samples approaching the lower limit of quantification (e.g., ICAM-3 in urine samples).

The graphs below show the average % recovery for each analyte at each dilution.

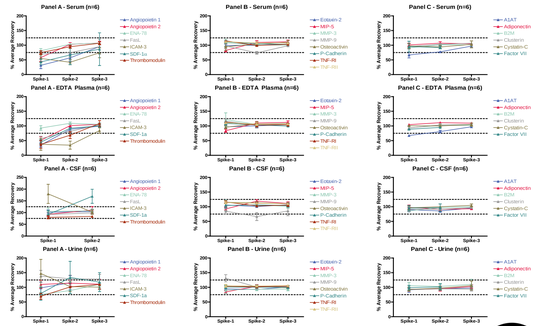
% Recovery = (measured x dilution factor)/expected x 100



5 Spike Recovery

Six normal human serum, EDTA plasma, CSF, and urine samples were spiked with calibrators at multiple levels throughout the linear range of the assay. Spikes were made into neat or diluted samples; the selected dilution factor was determined from the dilution linearity. Most of the analytes recovered between 75% and 125% at each spike level. For Panel A, under-recovery was observed for several analytes; further dilution optimization is recommended for these analytes.

The graphs below show the average % recovery for each analyte at each spike level.



% Recovery = measured/expected x 100

Panel A

	Spike Concentration			
Spot	Spike-1	Spike-2	Spike-3	Units
Angiopoietin 1	40.0	1.60	0.06	ng/mL
Angiopoietin 2	4000	160	6.40	pg/mL
ENA-78	500	20.0	0.80	pg/mL
FasL	1000	40.0	1.60	pg/mL
ICAM-3	80.0	3.20	0.13	ng/mL
SDF-1α	8000	320	12.8	pg/mL
Thrombomodulin	80.0	3.20	0.13	ng/mL

Panel B

	Spike Concentration			
Spot	Spike-1	Spike-2	Spike-3	Units
Eotaxin-2	500	31.3	1.95	pg/mL
MIP-5	2.50	0.16	0.01	ng/mL
MMP-3	25.0	1.56	0.10	ng/mL
MMP-9	125	7.81	0.49	ng/mL
Osteoactivin	10.0	0.63	0.04	ng/mL
P-Cadherin	25.0	1.56	0.10	ng/mL
TNF-RI	2.50	0.16	0.01	ng/mL
TNF-RII	625	39.1	2.44	pg/mL

Panel C

	Spike Concentration			
Spot	Spike-1	Spike-2	Spike-3	Units
A1AT	50.0	5.56	0.62	ng/mL
Adiponectin	6670	741	82.3	pg/mL
B2M	539	59.9	6.65	pg/mL
Clusterin	66.7	7.41	0.82	ng/mL
Cystatin-C	13.3	1.48	0.17	ng/mL
Factor VII	568	63.1	7.01	pg/mL

6 Specificity

To determine the specificity of the detection antibodies, blended calibrators were tested with each detection antibody. The non-specific interactions were below 1.0% for most analytes.

$$\%\text{Non} - \text{specificity} = \frac{Background\ subtracted\ non-specific\ signal}{Background\ subtracted\ specific\ signal} * 100$$

Panel A

	Angiopoietin 1	Angiopoietin 2	ENA-78	FasL	ICAM-3	SDF-1α	Thrombomodulin
Calibrator Conc. Tested (pg/mL)	40000	4000	500	1000	80000	8000	80000

	Blended Calibrator with Individual Detectors						
Spot	Angiopoietin 1	Angiopoietin 2	ENA-78	FasL	ICAM-3	SDF-1α	Thrombomodulin
Angiopoietin 1	100%	< 1.0%	< 1.0%	< 1.0%	5.5%	< 1.0%	< 1.0%
Angiopoietin 2	2.3%	100%	< 1.0%	< 1.0%	2.1%	< 1.0%	3.2%
ENA-78	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
FasL	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%
ICAM-3	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%
SDF-1α	1.6%	1.5%	1.2%	< 1.0%	5.2%	100%	< 1.0%
Thrombomodulin	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%

Panel B

	Eotaxin-2	MIP-5	MMP-3	MMP-9	Osteoactivin	P-Cadherin	TNF-RI	TNF-RII
Calibrator Conc. Tested (pg/mL)	500	2500	25000	125000	10000	25000	2500	625

	Blended Calibrator with Individual Detectors							
Spot	Eotaxin-2	MIP-5	MMP-3	MMP-9	Osteoactivin	P-Cadherin	TNF-RI	TNF-RII
Eotaxin-2	100%	< 1.0%	< 1.0%	1.5%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
MIP-5	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
MMP-3	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
MMP-9	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
Osteoactivin	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%
P-Cadherin	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%
TNF-RI	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%
TNF-RII	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%

Panel C

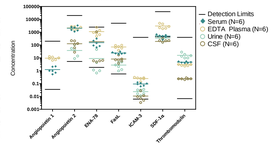
	A1AT	Adiponectin	B2M	Clusterin	Cystatin-C	Factor VII
Calibrator Conc. Tested (pg/mL)	50000	6667	539	66667	13333	568

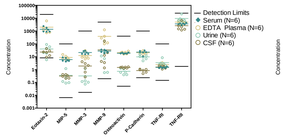
	Blended Calibrator with Individual Detectors					
Spot	A1AT	Adiponectin	B2M	Clusterin	Cystatin-C	Factor VII
A1AT	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
Adiponectin	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
B2M	< 1.0%	1.9%	100%	< 1.0%	1.5%	< 1.0%
Clusterin	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%
Cystatin-C	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%
Factor VII	7.3%	2.7%	< 1.0%	3.6%	2.5%	100%

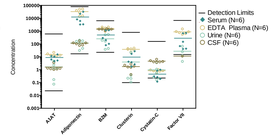
7 Sample Testing

Six normal human serum, EDTA plasma, urine, and CSF samples were tested across all three multiplex panels. The majority of samples measured above the lower limit of detection (LLOD). The table below provides the limits of detection and the concentration units for different assays.

The LLOD is a calculated concentration based on a signal that is 2.5 standard deviations over the blank. The median LLOD is calculated over 6 runs. The upper limit of detection (ULOD) is the highest calibrator concentration. Detection limits are reported at their dilution-adjusted concentration.







Assay	Panel	Dilution	Median LLOD	Median ULOD	Units
A1AT	C	4000	0.02	600	µg/mL
Adiponectin	C	4000	17.3	80000	ng/mL
Angiopoietin 1	A	2	0.04	200	ng/mL
Angiopoietin 2	A	2	5.30	20000	pg/mL
B2M	C	4000	22.5	6464	ng/mL
Clusterin	C	4000	0.10	800	µg/mL
Cystatin-C	C	4000	0.23	160	µg/mL
ENA-78	A	2	1.83	2500	pg/mL
Eotaxin-2	B	10	8.17	20000	pg/mL
Factor VII	C	4000	15.1	6816	ng/mL
FasL	A	2	0.57	5000	pg/mL
ICAM-3	A	2	0.01	400	ng/mL
MIP-5	B	10	0.01	100	ng/mL
MMP-3	B	10	0.02	1000	ng/mL
MMP-9	B	10	0.20	5000	ng/mL
Osteoactivin	B	10	0.05	400	ng/mL
P-Cadherin	B	10	0.23	1000	ng/mL
SDF-1α	A	2	438	40000	pg/mL
Thrombomodulin	A	2	0.01	400	ng/mL
TNF-RI	B	10	0.15	100	ng/mL
TNF-RII	B	10	1.77	25000	pg/mL

8 Conclusion

Fit-for-Purpose multiplex panels were developed for a set of 21 analytes on the U-PLEX platform. Assay feasibility was determined by testing for specificity, spike recovery, and dilution linearity. Relative sample abundance required the analytes to be separated into focused multiplex panels. The U-PLEX platform enables the easy reconfiguration of multiplex panels depending on the need.

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