Fit-for-Purpose Multiplex Panels and Their Utility in Biomarker Screening

D. Russell, J. Lewis, E. Spang, P. Oberoi, and J.N. Wohlstadter
Meso Scale Discovery, Rockville, Maryland, USA

❶ Abstract

Introduction: Exploratory studies to identify biomarkers important to disease may include screening for 100+ biomarkers. Identification of candidates can be misleading due to interference from large multiplex panels. In this study, smaller multiplex panels were evaluated for their utility in screening without a priori knowledge.

Materials and Methods: A biomarker screening panel was developed based on MSD's MULTI-ARRAY® technology, requiring <1 mL of sample to measure 122 analytes. The assays were grouped into 15 different multiplex panels following a fit-for-purpose approach. The dilution factors, diluent components, and specificity of reagents were optimized for each panel. Panels included MSD's analytically validated V-PLEX® Human Biomarker 40-Plex, which consists of biomarkers relevant to inflammation, immunology, angiogenesis, and vascular injury. The remaining assays were combined into multiplex panels of up to 10 assays.

Results: Multiplex panels were developed in a 10-plex format to facilitate optimization of assay protocols and performance. Assays typically exhibited <1.0% non-specific binding. The assay dynamic range was 3-4 orders of magnitude, enabling quantification of samples from both normal and diseased states. Patient sample sets included serum, EDTA-plasma, cerebrospinal fluid, and urine were measured. Individual assays had good reproducibility across plates. For the majority of the assays, the median intra-plate coefficient of variation (CV) was <10% across samples that were within the quantitative range of the assay.

Conclusions: Biomarker screening by an unbiased approach allowed rapid identification of targets of potential clinical significance.

❷ Methods

Samples were screened on a biomarker screening panel based on MSD's MULTI-ARRAY technology. Utilizing a fit-for-purpose methodology, 122 assays were grouped into 15 different multiplex panels. Dilution factors, diluent components, and specificity of reagents were optimized for each panel. Five of the panels were comprised of assays from MSD's analytically validated V-PLEX Human Biomarker 40-Plex Kit. The remaining assays were combined into multiplex panels of up to 10 assays. Less than one mL of sample was required to measure all 122 analytes.
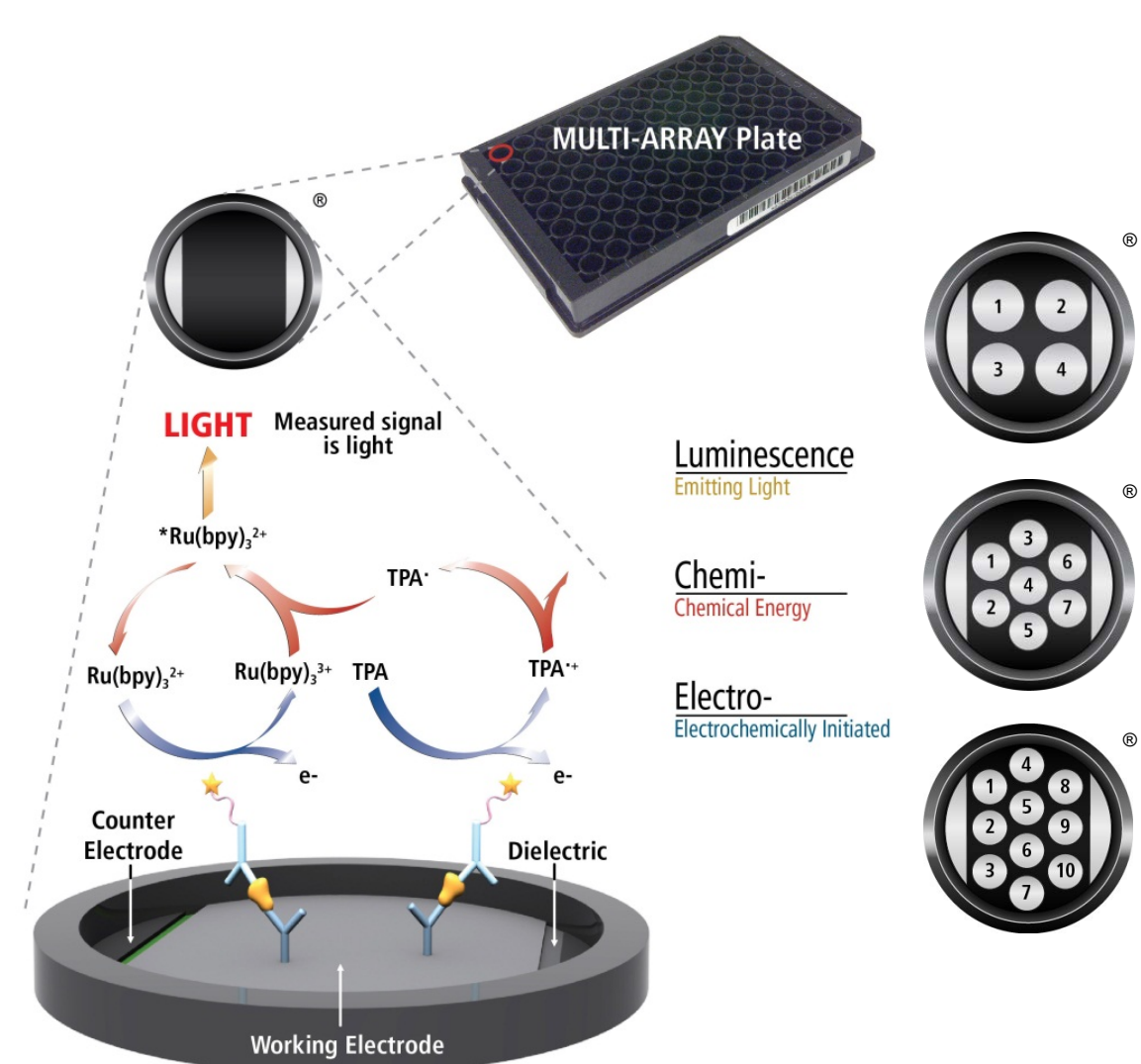
Electrochemiluminescence Technology

- 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.

❸ Specificity

To determine detection antibody specificity, blended calibrators were tested with individual detection antibodies. Testing was conducted for each of the 15 panels. We found that non-specific interactions were below 1.0% for most analytes. Representative data is shown below.

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

	Calbindin	Eotaxin-2	MIP-5	MMP-1	MMP-3	MMP-9	Osteoactivin	P-Cadherin	TNF-RI	TNF-RII
Calibrator Conc. Tested (pg/mL)	6250	500	2500	25000	25000	125000	10000	25000	2500	625

Blended Calibrator with Individual Detectors										
Spot	Calbindin	Eotaxin-2	MIP-5	MMP-1	MMP-3	MMP-9	Osteoactivin	P-Cadherin	TNF-RI	TNF-RII
Calbindin	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
Eotaxin-2	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
MIP-5	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
MMP-1	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
MMP-3	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
MMP-9	< 1.0%	< 1.0%	< 1.0%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%	< 1.0%	< 1.0%
Osteoactivin	< 1.0%	< 1.0%	1.1%	< 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%	< 1.0%	< 1.0%	100%	< 1.0%	< 1.0%
TNF-RI	< 1.0%	< 1.0%	< 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%	< 1.0%	< 1.0%	100%

❹ Sensitivity

The lower limit of detection (LLOD) is a calculated concentration based on a signal that is 2.5 standard deviations over the blank. At least 6 runs were used to calculate the median LLOD. The upper limit of detection (ULOD) is the highest calibrator concentration. Detection limits are reported at their dilution-adjusted concentrations in the table below.

Most assays tested used the same dilution factor for all matrices. CRP, ICAM-1, SAA, and VCAM-1 are tested at a 1000-fold dilution for serum and plasma, and at a 5-fold dilution for CSF and urine.

Assay	Dilution	Median LLOD	Median ULOD	Units
A2M	4000	0.26	2700	µg/mL
Active GLP-1	2	0.20	1000	pg/mL
Adiponectin	4000	6.7	80000	ng/mL
Angiopoietin 1	2	0.099	200	ng/mL
Angiopoietin 2	2	5.0	20000	pg/mL
B2M	4000	1.6	6500	ng/mL
CA 125	20	0.0034	100	kIU/mL
CA 15.3	20	0.33	20000	mIU/mL
CA 50	20	79	80000	mIU/mL
Calbindin	10	0.23	250	ng/mL
CEA	20	0.19	2000	pg/mL
c-Kit	20	4.0	3000	ng/mL
CKMB	4	88	2200	ng/mL
Clusterin	4000	0.059	800	µg/mL
C-Peptide	2	28	50000	pg/mL
CRP	1000	0.0026	290	µg/mL
CTACK	4	28	60000	pg/mL
cTnI	4	0.0076	100	ng/mL
cTnT	4	0.95	200	ng/mL
Cytokeratin-8	2	0.44	2000	ng/mL
E-Cadherin	20	0.077	4000	ng/mL
ENA-78	2	0.91	2500	pg/mL
Endoglin	50	0.038	500	ng/mL
Eotaxin	4	6.4	6100	pg/mL
Eotaxin-2	10	3.2	20000	pg/mL
Eotaxin-3	4	8.2	19000	pg/mL
EPO	2	1.7	10000	mIU/mL
E-Selectin	2	0.053	400	ng/mL
FABP3	4	0.25	400	ng/mL
Factor VII	4000	7.0	6800	pg/mL
Fas	50	0.062	250	ng/mL
FasL	2	0.85	5000	pg/mL
FGF (basic)	2	0.17	4100	pg/mL
Flt-1	2	1.3	16000	pg/mL
Flt-3 Ligand	20	0.71	60000	pg/mL
Fractalkine	4	0.10	400	ng/mL
GIP	2	4.9	2500	pg/mL
Glucagon	2	29	10000	pg/mL
GM-CSF	2	0.27	1900	pg/mL
GRO-α	4	14	10000	pg/mL
I-309	4	0.28	1000	pg/mL
ICAM-1	1000	1.4	69000	ng/mL
ICAM-3	2	0.0040	400	ng/mL
IFN-α	2	2.1	10000	pg/mL
IFN-γ	2	0.67	2100	pg/mL
IL-10	2	0.060	630	pg/mL
IL-12/IL-23p40	2	0.54	5800	pg/mL
IL-12p70	2	0.27	810	pg/mL
IL-13	2	1.7	990	pg/mL
IL-15	2	0.30	1400	pg/mL
IL-16	2	5.1	4900	pg/mL
IL-17	2	0.93	9500	pg/mL
IL-17B	2	9.6	5000	pg/mL
IL-17D	2	4.7	5000	pg/mL
IL-18	2	4.1	2500	pg/mL
IL-1Ra	50	0.046	250	ng/mL
IL-1α	2	0.13	670	pg/mL
IL-1β	2	0.16	1000	pg/mL
IL-2	2	0.17	2800	pg/mL
IL-21	2	2.1	10000	pg/mL
IL-33	2	2.7	1500	pg/mL

Assay	Dilution	Median LLOD	Median ULOD	Units
IL-4	2	0.056	390	pg/mL
IL-5	2	0.19	1600	pg/mL
IL-6	2	0.66	1500	pg/mL
IL-6R	50	0.010	500	ng/mL
IL-7	2	0.27	1400	pg/mL
IL-8	2	0.13	1000	pg/mL
Insulin	2	36	50000	pg/mL
IP-10	4	0.36	11000	pg/mL
I-TAC	4	2.3	10000	pg/mL
Leptin	2	0.053	100	ng/mL
MCP-1	4	0.28	2000	pg/mL
MCP-2	2	2.1	10000	pg/mL
MCP-3	4	0.48	2500	pg/mL
MCP-4	4	5.0	2600	pg/mL
M-CSF	2	0.072	2500	pg/mL
MDC	4	11	41000	pg/mL
Mesothelin	50	0.068	1200	ng/mL
Met	20	0.098	4000	ng/mL
MIF	2	27	60000	pg/mL
MIG	4	1.4	10000	pg/mL
MIP-1α	4	3.4	4200	pg/mL
MIP-1β	4	1.5	4400	pg/mL
MIP-3α	4	0.19	2500	pg/mL
MIP-3β	4	1.1	40000	pg/mL
MIP-5	10	0.0050	100	ng/mL
MMP-1	10	0.039	1000	ng/mL
MMP-3	10	0.076	1000	ng/mL
MMP-9	10	0.16	5000	ng/mL
Myl3	4	0.17	220	ng/mL
Myoglobin	4	17	40000	ng/mL
Nectin-4	2	0.52	20000	pg/mL
NT-proBNP	4	8.6	20000	pg/mL
Osteoactivin	10	0.16	400	ng/mL
Osteocalcin	50	2.1	10000	ng/mL
Osteonectin	2	0.69	2000	ng/mL
Osteopontin	20	14	4000	ng/mL
Osteoprotegerin	2	0.010	200	ng/mL
P-Cadherin	10	0.099	1000	ng/mL
PlGF	2	0.53	7100	pg/mL
P-Selectin	2	0.13	400	ng/mL
PYY (total)	2	9.3	3000	pg/mL
RANTES	50	0.012	500	ng/mL
Resistin	50	0.016	130	ng/mL
SAA	1000	0.018	240	µg/mL
SCF	2	0.36	10000	pg/mL
SDF-1α	2	870	40000	pg/mL
TARC	4	1.1	6300	pg/mL
Tenascin C	4000	2.8	690	ng/mL
Thrombomodulin	2	0.0041	400	ng/mL
Tie-2	2	0.038	160	ng/mL
TNF-RI	10	0.032	100	ng/mL
TNF-RII	10	1.4	25000	pg/mL
TNF-α	2	0.12	640	pg/mL
TNF-β	2	0.079	1200	pg/mL
TPO	4	4.4	40000	pg/mL
TRAIL	2	0.41	20000	pg/mL
VCAM-1	1000	6.3	70000	ng/mL
VEGF-A	2	0.93	2000	pg/mL
VEGF-C	2	27	44000	pg/mL
VEGF-D	2	5.3	47000	pg/mL
YKL-40	50	0.038	2500	ng/mL

❺ Sample Testing

Twenty human serum, 20 EDTA plasma, 8 urine, and 8 CSF samples were tested across the 15 panels. For the majority of assays, samples were detectable. IL-17B, IL-17D, and IL-21 were not detectable in normal samples.

Concentration units are listed on the left in the table with the limits of detection.
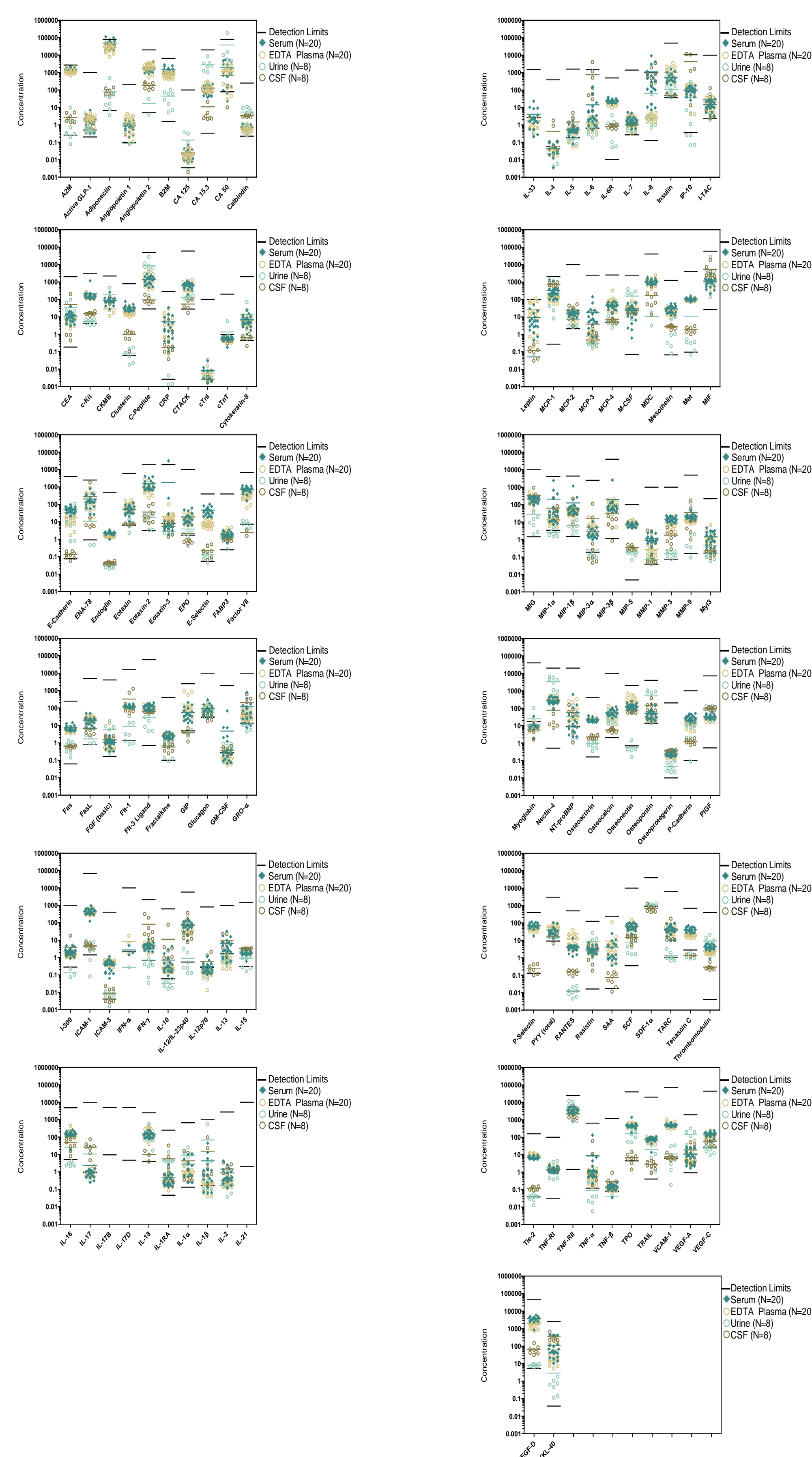
❻ Reproducibility

The median CV was calculated for samples within the limits of quantification. For assays included in the V-PLEX Human Biomarker 40-Plex, the lower and upper limits of quantification (LLOQ and ULOQ, respectively) were obtained from the certificate of analysis for the kit. For the additional assays, the limits of quantification were estimated. The LLOQ was estimated as 5 times the median LLOD. The ULOQ was estimated as 80% of the ULOD.

For serum, EDTA plasma, and CSF, at least 90% of the assays had a median CV of less than 10%. For urine, 70% of the assays had a median CV of less than 10%.
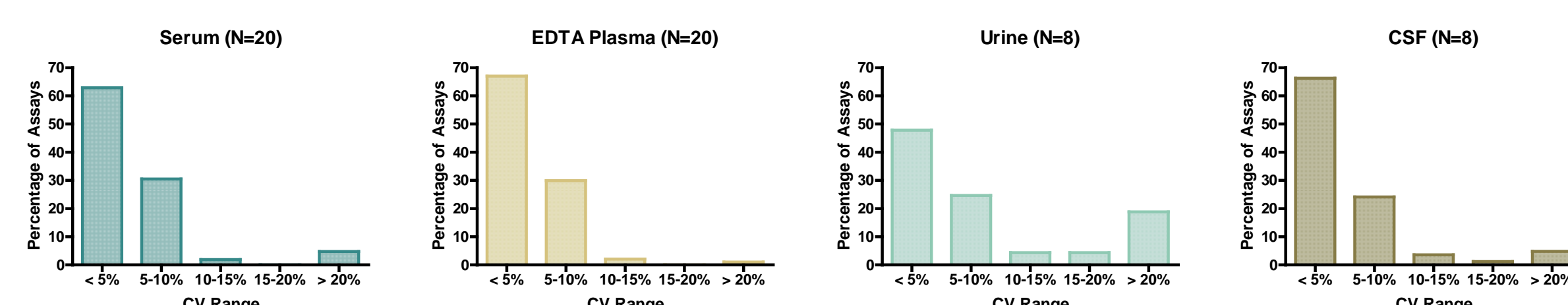

Elevated CVs often correlated with low endogenous levels (see Section 5). The following assays had a median CV of greater than 20%:
- Serum – IL-2, IL-13, IL-17, PYY (total), and Osteopontin.
- EDTA Plasma – Eotaxin-3.
- CSF – IL-2, IL-4, IL-13, and RANTES.
- Urine – IL-1α, VEGF-A, IL-16, C-Peptide, E-Selectin, NT-proBNP, cTnT, Myl3, CKMB, Myoglobin, Osteoprotegerin, MCP-2, and MET.

The median CV was calculated for the standard curve. The median signal CV was less than 10% for 98% of the assays at standard five concentration (Cal-5).

The CKMB assay had a median CV of greater than 20%. This assay was further optimized to expand the dynamic range of the assay (data not shown).

Reproducibility (precision) was assessed with matrix-based controls tested across 6 plates on a single day of testing. Representative data is shown below.
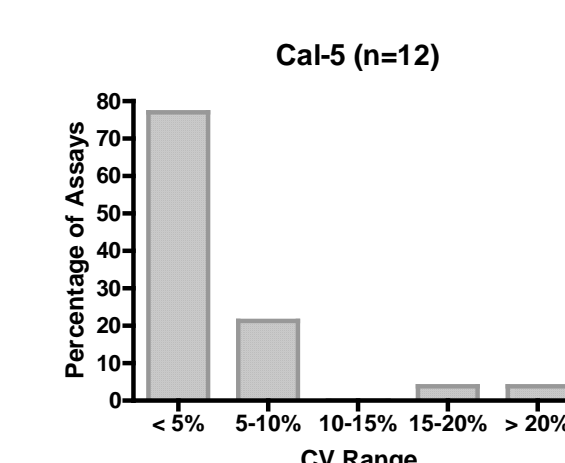
Assay	Sample	Runs	Avg Conc.	Units	Avg Intra-plate %CV	Inter-plate %CV
A2M	Sample 1	6	1170	µg/mL	3.3	5.0
	Sample 2	6	1219	µg/mL	7.6	9.3
	Sample 3	6	2391	µg/mL	9.1	11.1
	Sample 4	6	1149	µg/mL	2.4	8.5
Adiponectin	Sample 1	6	54704	ng/mL	5.1	5.5
	Sample 2	6	50878	ng/mL	8.5	7.1
	Sample 3	6	64417	ng/mL	4.5	5.0
	Sample 4	6	21521	ng/mL	4.2	3.9
Clusterin	Sample 1	6	26.1	µg/mL	5.0	6.5
	Sample 2	6	20.4	µg/mL	8.4	9.8
	Sample 3	6	9.82	µg/mL	5.1	5.6
	Sample 4	6	27.0	µg/mL	6.1	7.4
Factor VII	Sample 1	6	400	ng/mL	4.2	4.5
	Sample 2	6	440	ng/mL	4.8	5.1
	Sample 3	6	361	ng/mL	3.1	2.9
	Sample 4	6	888	ng/mL	3.9	4.6
FGF (basic)	Sample 1	6	1885	pg/mL	2.8	4.2
	Sample 2	6	194	pg/mL	5.6	6.0
	Sample 3	6	21.2	pg/mL	5.9	6.4
	Sample 4	6	1.88	pg/mL	5.1	6.2
Flt-1	Sample 1	6	6560	pg/mL	1.8	2.9
	Sample 2	6	698	pg/mL	1.8	2.3
	Sample 3	6	68.1	pg/mL	4.1	5.5
	Sample 4	6	79.3	pg/mL	4.9	6.4
PlGF	Sample 1	6	2961	pg/mL	6.1	7.4
	Sample 2	6	324	pg/mL	5.7	6.7
	Sample 3	6	36	pg/mL	6.9	9.7
	Sample 4	6	26.7	pg/mL	2.7	7.5
Tenascin C	Sample 1	6	37.6	ng/mL	4.8	11.1
	Sample 2	6	34.4	ng/mL	9.6	11.6
	Sample 3	6	29.4	ng/mL	13.3	13.7
	Sample 4	6	35.6	ng/mL	3.6	13.8
Tie-2	Sample 1	6	67.7	ng/mL	3.7	4.9
	Sample 2	6	8.82	ng/mL	3.5	10.7
	Sample 3	6	2.05	ng/mL	4.0	3.4
	Sample 4	6	11.8	ng/mL	5.4	12.3

❼ Conclusion

MSD's Biomarker Screening Panel includes a robust menu of assays that quantitatively measure relevant biomarkers. The assays have a broad dynamic range that allows scientists to obtain an accurate quantification of their samples. For most assays, multiple sample types can be tested at a single dilution factor. The panel uses simple protocols with measurement of up to 10 biomarkers within a plate. The 122 biomarkers were measured using less than 1 mL of sample. The panel may be used to quantify the levels of biomarkers in a variety of matrices including serum, plasma, CSF, and urine.

Meso Scale Discovery
A division of Meso Scale Diagnostics, LLC.
www.mesoscale.com®