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Development and Validation of a Human Biomarker 40-Plex V-PLEX® Panel – A Tool for Identifying Biomarkers in Health and Disease

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1 Abstract

Purpose: The identification and validation of protein biomarkers that can accurately report biological function or phenotype is necessary for advancing drug discovery and diagnostic capabilities. While many biomarker assays are available, their lack of analytical validation and lot-to-lot reproducibility remains a barrier to the success of long-term biomarker programs. These challenges can be addressed with larger multiplex panels that have been validated and designed to facilitate biomarker discovery in research programs.
Methods: We developed and validated 40 assays for biomarkers relevant to inflammation, immunology, angiogenesis, and vascular injury. These assays detect IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNFα, GM-CSF, IL-1α, IL-5, IL-7, IL-17A, IL-15, IL-16, IL-12/IL-23p40, TNFβ, IL-8 (high abundance), MCP-1, MDC, MCP-4, Eotaxin, IP-10, Eotaxin-3, TARC, MIP-1α, MIP-1β, VEGF, VEGF-C, VEGF-D, Tie-2, sFlt1, PlGF, bFGF, SAA, CRP, VCAM-1 and ICAM-1. All assays were developed with highly characterized and qualified reagents in accordance with fit-for-purpose industry guidelines. Diluents, other kit components, and an optimized assay protocol were developed to help achieve optimum performance. The 40-plex panel rapidly quantified these biomarkers with high precision and accuracy in different human matrices.
Results: We present the assay validation procedure and reproducibility data within and across lots for all 40 assays using a minimum of 3 production lots (generated from independent component lots when possible). The data demonstrate that more than 80% of the assays are sensitive enough to detect low abundance biomarkers in normal biological matrices such as serum, plasma, and urine. Some of the low abundance analytes are quantified at levels below 0.5 pg/ml. In general, these assays display less than 1% non-specific binding with other analytes in the panel or related analytes not in the panel. In addition, we show that subsets of these biomarkers can be multiplexed in any combination without compromising performance.
Conclusions: The Human Biomarker 40-Plex V-PLEX Panel provides an accurate and reproducible tool for the identification of a wide array of biomarkers suitable for research or drug development programs. A summary of the development and validation of these panels is presented.

2 Methods

The 40-plex panel was developed as five individual panels designed for optimal performance based on individual assay characteristics, native analyte levels and suitable dilution, diluent, and assay compatibility. The assays presented were developed and validated following fit-for-purpose principles and design control procedures. Accuracy, precision, and specificity were evaluated by multiple operators using independently built kit lots to test calibrators, controls, and matrix-based samples over multiple days and runs. The Pro-inflammatory Panel 1 (human), Cytokine Panel 1 (human), Chemokine Panel 1 (human), Angiogenesis Panel 1 (human), and Vascular Injury Panel 2 (human) kits are based on MSD® ECL technology and follow the same simple three-step protocol

Electrochemiluminescence Technology

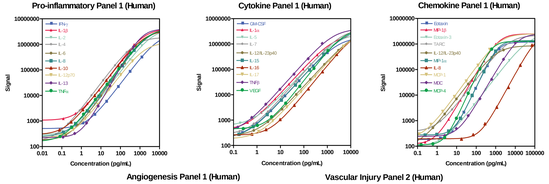
- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed 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.

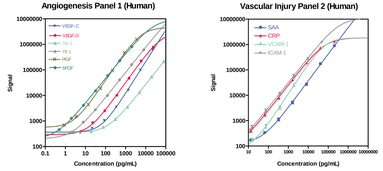
Protocol

1. Add calibrator, control or sample (25 µL/well). Incubate 2 hours at room temperature (RT).
2. Wash and add detection antibody solution (25 µL/well). Incubate 1 hour at RT.
3. Wash and add read buffer (150 µL/well). Analyze with MSD instrument.

3 Calibration Curves

Representative calibration curves are shown below for each of the 40 assays.





4 Sensitivity

The lower limit of detection (LLOD) is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator). Limits of quantification (LOQ) were determined by spiking a known value of calibrator into diluent to assess the accuracy and precision of the samples across multiple lots. The lower LOQ (LLOQ) is the lowest concentration at which the CV of the calculated concentration is <20% and the recovery of the analyte is within 80% to 120% of the known value (75% to 125% for IL-8 in the Chemokine Panel 1 (Human) Kit). The upper LOQ (ULOQ) is the highest concentration at which the CV of the calculated concentration is <20% and the recovery of the analyte is within 80% to 120% of the known value (75% to 125% for IL-8 in the Chemokine Panel 1 (Human) Kit). The data are from >30 runs collected over 2 months by 4 operators.

Pro-inflammatory Panel 1 (Human)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
IFN-γ	0.20	7.47	938
IL-1β	0.04	2.14	375
IL-2	0.09	0.89	938
IL-4	0.02	0.45	158
IL-6	0.06	1.58	488
IL-8	0.04	1.13	375
IL-10	0.03	0.68	233
IL-12p70	0.11	1.22	315
IL-13	0.24	4.21	353
TNFα	0.04	0.69	248

Cytokine Panel 1 (Human)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
GM-CSF	0.14	1.90	750
IL-1α	0.09	2.85	278
IL-5	0.22	6.28	562
IL-7	0.16	1.37	563
IL-12/IL-23p40	0.39	5.68	2250
IL-15	0.17	1.40	525
IL-16	2.83	19.1	1875
IL-17A	0.74	9.32	3653
TNFβ	0.05	1.15	458
VEGF	1.12	7.70	784

Chemokine Panel 1 (Human)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
Eotaxin	3.26	12.3	1120
MIP-1β	0.37	2.27	750
Eotaxin-3	1.77	10.2	3750
TARC	0.22	3.32	1120
IP-10	0.37	1.37	500
MIP-1α	3.02	13.8	743
IL-8*	95.6	713	43400
MCP-1	0.09	1.09	375
MDC	1.22	88.3	7500
MCP-4	1.69	5.13	469

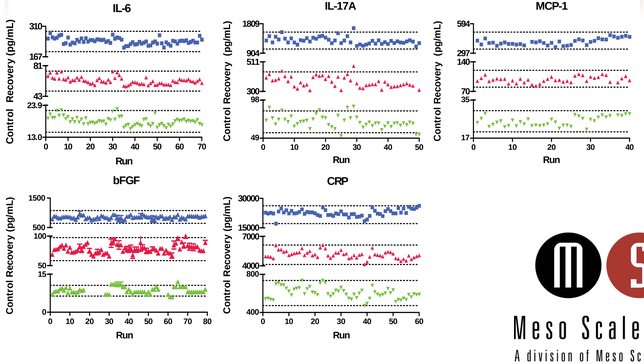
*Because of the high abundance of IL-8 in some sample types, Chemokine Panel 1 (Human) uses a low sensitivity IL-8 assay.

Angiogenesis Panel 1 (Human)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
VEGF-C	11.1	146	17500
VEGF-D	2.53	67.1	18800
Tie-2	21.1	396	63400
Flt-1	0.56	10	6410
PlGF	0.32	10.7	2370
bFGF	0.08	2.6	1780

Vascular Injury Panel 2 (Human)			
Analyte	LLOD Median (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
SAA	10.9	54.1	137972
CRP	1.33	27.6	49608
VCAM-1	6.00	37.6	31960
ICAM-1	1.03	6.4	42708

5 Specificity and Reproducibility

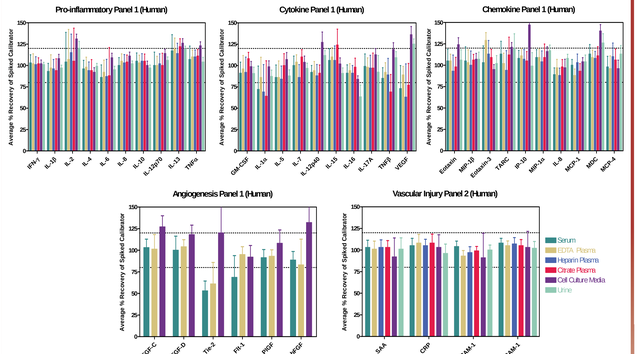
Each assay in the 40-plex panel was evaluated for cross-reactivity with other analytes in the panel as well as related analytes not in the panel. The cross-reactivity for all assays was <1% (data not shown).
To evaluate the reproducibility of the panels, three levels of controls spanning the quantifiable range were prepared and measured over multiple runs for each panel. The measured concentrations for a representative assay from each of the 10-plex panels are shown. All controls were prepared in a non-human matrix except for Controls 1 and 2 for bFGF (Human EDTA Plasma) and CRP Controls (diluent). Between 40 and 80 runs were conducted depending on the panel. All controls tested were within the target range of ±20% of assigned concentration (dashed lines).



6 Spike Recovery and Dilution Linearity

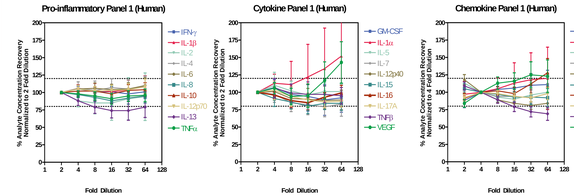
Spike recovery and dilution linearity were assessed across multiple lots. Spike recovery testing was conducted in serum, heparin plasma, EDTA plasma, citrate plasma, urine, and cell culture media. Representative data are presented below showing overall performance of each assay. Almost all assays accurately recovered expected concentrations of spiked analyte in each matrix (dashed lines are at ±20% of expected value).

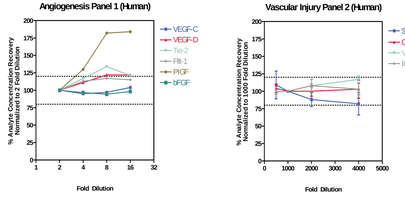
Spike Recovery



Dilution Linearity

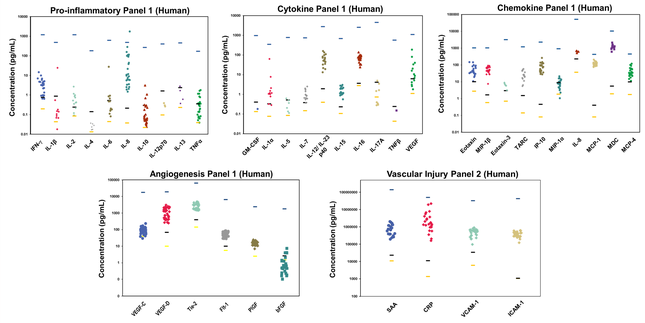
Dilution linearity testing was conducted on all assays. Results were similar with almost all samples recovering within 20% of expected concentrations across all assays. The one exception is PlGF whose performance may benefit from additional dilution.





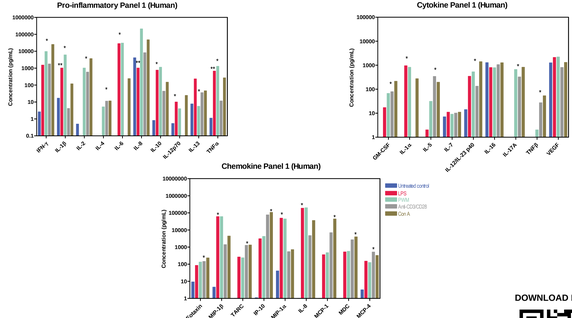
7 Normal Sample Testing

Normal human matrices (obtained from a commercial vendor) were evaluated for native analyte levels. The representative data for serum is shown with ULOQ (dark blue bars), LLOQ, (black bars) and LLOD (yellow bars) noted for each assay. Nearly all samples were detectable across the 40-plex and many samples were within the LOQ.



8 Whole Blood Testing

Peripheral blood lymphocytes were treated with various stimulants including LPS (10µg/ml), PWM (50µg/ml), anti-CD3/CD28 (5µg/ml each), or ConA (20µg/ml) for 24 hours. The stimulated samples were tested on Pro-inflammatory Panel 1 (human), Cytokine Panel 1 (human) and Chemokine Panel 1 (human) kits. Stimulated studies for the Angiogenesis Panel 1 (human) and the Vascular Injury Panel 2 (human) were not performed because they are well-represented in normal matrices such as serum. Results denoted with (*) are significant relative to controls, and bars denoted with (**) are concentrations above saturation levels. Dose dependent stimulation was observed for all analytes.



9 Conclusion

A 40-analyte panel has been developed and analytically validated for use in the quantification of biologically relevant cytokines and chemokines in multiple human matrices. All assays in these five multiplex panels were individually optimized to achieve the best assay performance and for ease of use, featuring the same simple 3-step protocol requiring just 25 µL of sample to measure all 40 analytes. Consistent results were demonstrated for calibration curves and control samples over multiple runs on different days by multiple operators. These assays have sufficient sensitivity, reproducibility, accuracy and performance in different matrices to facilitate biomarker discovery and validation in any research or drug development program.

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