#T2068

Development and Validation of a 10-plex Mouse Proinflammatory Cytokine V-PLEX® Panel

Joseph Manimala, Prachi Gupte, Amandeep Kaur, Ken Wong, Angelina Anderson, David Stewart, Pankaj Oberoi, and Jacob N. Wohlstadter

Meso Scale Discovery, Rockville, Maryland, USA

1 Abstract

Purpose: Multiplex cytokine assays are widely available; however, consistency and inter-lot reproducibility has been a challenge that is especially troublesome for long-term studies. Working groups from several consortia and research societies have expressed a need for standardization in the validation of these assays. We have developed a multiplex panel that allows optimal performance of 10 mouse cytokine assays and have validated this panel across multiple lots to demonstrate consistency within and between kit lots.

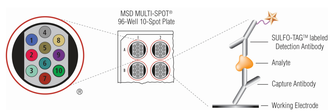
Methods: We developed and validated a mouse proinflammatory cytokine multiplex panel in accordance with fit-for-purpose and industry guidelines. The panel consists of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC, IL-10, IL-12p70, and TNF-α. It has the ability to simultaneously measure these biomarkers in serum, EDTA plasma, heparin plasma, citrate plasma, urine, or cell culture supernatant. All critical reagents were subjected to analytical characterization, and each kit component and method was optimized to achieve the desired performance. Each assay was evaluated for reproducibility within and across at least 3 lots of materials.

Results: We present the validation procedure and assay performance reproducibility data for the 10-plex panel across 3 lots generated from independent component lots. Most assays were sensitive enough to detect even very low abundance biomarkers in various normal biological matrices—including serum, plasma, and urine—using a 25 µl sample. Typical intra-run coefficients of variation (CVs) were <7%, and inter-lot and inter-run CVs were typically <10%. We present dilution linearity and spike recovery data for the validated matrices. The calibrator and sample measurements are reproducible within and across lots with most assays having a cross-correlation >0.9. In addition, we show that these assays can be multiplexed in any combination without compromising their performance.

Conclusions: The Proinflammatory Panel 1 (mouse) V-PLEX panel provides a suitable method for accurately quantitating up to 10 cytokines simultaneously in various sample types. The performance and consistency demonstrated through the multi-lot validation process supports the use of this tool for long-term studies in drug development and other applications.

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.



Electrochemiluminescence Technology

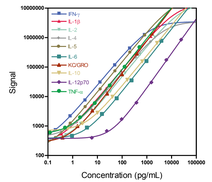
- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed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

Development: Proinflammatory Panel 1 (mouse) development included optimization of various assay parameters such as antibody concentration, diluent composition, and protocol timing. Calibration curve concentrations for each assay were optimized for maximum dynamic range while maintaining sufficient calibration points near the low end of the curve to ensure accurate quantification of samples requiring high sensitivity. The specificity of both capture and detection antibodies was measured during assay development. Antibody specificity was assessed by first running each assay individually on a multiplex plate with assay-specific detection antibody and assay-specific calibrator. In order to confirm the kit's ability to quantify analytes at concentrations higher than those often found in normal samples, whole blood, PBMCs, and/or cell line samples were stimulated to increase endogenous analyte levels. As part of development, real-time and accelerated stability testing was conducted for components and kits. Freeze-thaw and storage (2–8°C) stability were evaluated for both reconstituted calibrators and controls.

Validation: Proinflammatory Panel 1 (mouse) was validated following fit-for-purpose principles under MSD's design control system. Prior to the release of the product, 3 independent kit lots were produced and analytically validated in the final product configuration. Results from multiple runs (typically greater than 50 runs performed by multiple operators) were used to establish production specifications for sensitivity, specificity, accuracy, and precision. In addition, each assay in the multiplex panel was analytically validated on a single-spot plate. The panel was validated by evaluating sensitivity, quantifiable range, accuracy, precision, antibody specificity, spike recovery, and dilution linearity using 6 sample types (serum, EDTA plasma, heparin plasma, citrate plasma, urine, and cell culture supernatants) across 3 lots. Precision is reported as the coefficient of variation (CV). The validation program includes a real-time stability study of the Proinflammatory Panel 1 (mouse) kit with scheduled performance evaluations of complete kits for up to 54 months from the date of manufacture.

4 Calibration Curves



Representative average calibration curves from one kit lot are presented. Data were collected over 6 months of testing by 5 operators (63 runs in total). Most assays displayed a minimum 4-log dynamic range. At least 2 replicates of calibrators were run on each plate.

5 Assay Protocol, and Sensitivity

Protocol

1. Add 50 µL/well of calibrator, controls, or diluted samples; incubate 2 hours at room temperature (RT).
2. Wash; add 25 µL/well of detection antibody solution; incubate 2 hours at RT.
3. Wash; add 150 µL/well of Read Buffer T; read plate on MSD® instrument.

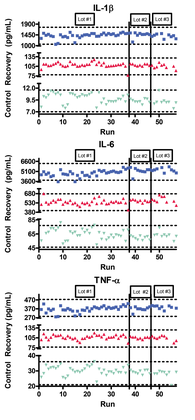
Lot	Lot #1		Lot #2			Lot #3		
Limits (pg/mL)	Median LLOD (N=63)	Median LLOD (N=31)	LLOQ	ULOQ	Median LLOD (N=25)	LLOQ	ULOQ
IFN-γ	0.04	0.04	0.39	724	0.02	0.40	734
IL-1β	0.11	0.09	0.72	1320	0.07	0.72	1320
IL-2	0.22	0.24	1.09	2020	0.19	1.03	1900
IL-4	0.14	0.16	0.71	1320	0.11	2.58	1190
IL-5	0.07	0.05	0.41	753	0.04	1.60	740
IL-6	0.63	0.69	7.05	3260	0.60	6.82	3140
KC/GRO	0.24	0.26	3.18	1470	0.17	3.01	1390
IL-10	1.06	0.86	5.2	2400	0.69	4.96	2290
IL-12p70	9.52	11.7	47.3	21800	7.27	44.7	20600
TNF-α	0.13	0.21	0.98	454	0.18	0.99	457

The calculated LLOD, LLOQ, and ULOQ are shown in the table above. The quantitative range of the assay lies between the LLOQ and ULOQ. LLOQ and ULOQ samples were tested with two lots of kits during validation. The LLOQ and ULOQ are verified for each production lot, and the results are provided in the lot-specific C of A.

6 Reproducibility

Precision and accuracy statistics for controls with dilution-adjusted concentrations reported in pg/mL.

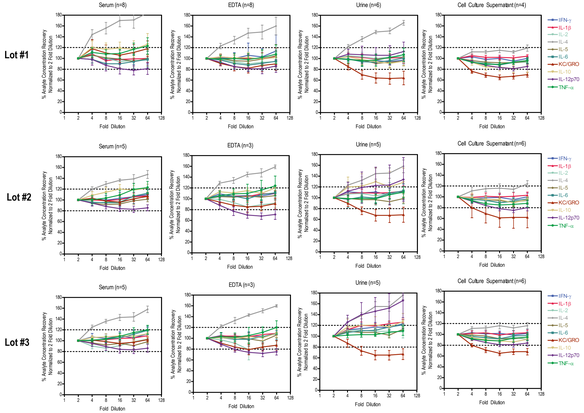
		Lot 1 (N=37)			Lot 2 (N=9)			Lot 3 (N=9)			Inter-lot	
Analyte	Control	Av. Conc.	Av. Intra-run %CV	Inter-run %CV	Av. Conc.	Av. Intra-run %CV	Inter-run %CV	Av. Conc.	Av. Intra-run %CV	Inter-run %CV	Av. Inter-lot Conc.	Inter-lot %CV
IFN-γ	A00C0160	737	6.0	13.3	776	4.3	8.2	707	2.8	5.7	740	4.7
	A00C0161	58.8	2.0	8.1	57.5	2.7	7.7	51.9	2.0	7.9	56.1	6.5
	A00C0162	5.37	2.5	12.3	5.10	2.9	8.5	4.63	1.7	7.1	5.03	7.4
IL-1β	A00C0160	1417	2.3	8.2	1450	3.6	8.0	1368	2.9	5.9	1412	2.9
	A00C0161	110	1.1	7.0	106	2.4	8.2	98.6	2.5	6.9	105	5.5
	A00C0162	10.0	1.6	11.2	9.31	2.7	9.2	8.62	2.6	11.1	9.31	7.4
IL-2	A00C0160	2519	1.6	8.6	2647	2.9	8.3	2346	3.0	4.5	2504	6.0
	A00C0161	200	1.6	7.9	193	2.9	8.7	168	3.3	9.1	187	9.0
	A00C0162	15.6	1.9	12.2	15.0	3	8.3	13.8	3.9	10.9	14.8	6.2
IL-4	A00C0160	737	2.5	7.3	697	2.8	7.8	669	2.3	4.2	701	4.9
	A00C0161	78.3	2	7.7	74.3	2.5	6.4	69.3	2.7	8.1	74.0	6.1
	A00C0162	11.7	2.5	11.1	10.7	3.1	10.9	9.60	3.6	8.4	10.7	9.8
IL-5	A00C0160	858	2.5	9.3	867	2.8	8.6	772	3.0	7.3	832	6.3
	A00C0161	57.2	1.9	9.4	54.0	2.6	8.3	48.5	2.3	10.0	53.2	8.3
	A00C0162	3.04	2.8	16.3	2.81	2.8	9.2	2.78	3.7	11.5	2.88	4.9
IL-6	A00C0160	4751	2.2	11.2	5165	2.3	9.5	5179	2.4	6.1	5032	4.8
	A00C0161	538	1.7	7.4	539	2.5	8.3	549	3.5	9.3	542	1.1
	A00C0162	65.2	1.6	11.3	59.4	3	7.9	59.8	3.1	7.8	61.5	5.3
KC/GRO	A00C0160	1961	1.6	10.6	1909	2.5	10.9	1896	2.5	4.5	1922	1.8
	A00C0161	250	1.6	7.8	241	2	8.1	219	2.7	6.2	237	6.7
	A00C0162	28.0	3.0	10.5	25.5	2.5	9.7	23.1	2.6	5.8	25.5	9.6
IL-10	A00C0160	2897	3.4	8.9	2553	5.3	8.1	2741	3.5	4.5	2730	6.3
	A00C0161	661	2.4	8.6	573	6.4	7.6	624	2.9	9.7	619	7.1
	A00C0162	146	2.5	9.7	131	6.4	10.9	133	3.8	8.8	137	6.0
IL-12p70	A00C0160	31 936	2.2	12.6	33 910	2.1	10.4	32 636	2.4	6.8	32 794	3.1
	A00C0161	4282	1.6	12.1	4762	2.1	10.9	4439	1.8	10.7	4494	5.4
	A00C0162	683	1.7	10.4	641	2.3	9.6	604	2.8	10.9	643	6.2
TNF-α	A00C0160	364	1.8	9.6	369	3.1	6.8	406	3.5	5.0	380	6.0
	A00C0161	107	1.4	6.3	103	2.9	6.6	100	2.1	7.1	103	3.4
	A00C0162	30.4	1.8	9.4	29.3	2.8	8.6	25.7	3.0	10.3	28.5	8.6



The accuracy and precision of each assay was assessed using calibrators and control samples. The back-fit concentrations of all calibrator points within the quantifiable range of each assay yielded CVs below 20% and recoveries within 80% to 120% of expected concentration (data not shown). A minimum of 5 calibrator points are within the quantifiable range of each assay. The precision and accuracy statistics for controls are listed above. For this panel, the data shows that the CVs for most assays are below 10%. The reported concentration was dilution adjusted to the concentration of the controls upon initial reconstitution in 250 µL of Diluent 41.

Dilution-adjusted control recoveries for each run (control lots A00C0160, A00C0161, and A00C0162) for selected assays are plotted above for 3 kit lots. The specification for control recovery is a concentration within 75% to 125% of the average multi-lot concentration (dashed lines).

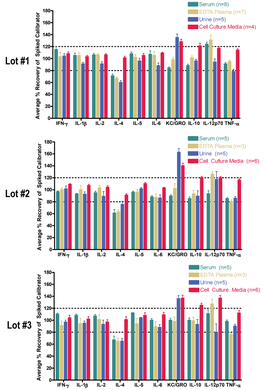
7 Dilution Linearity



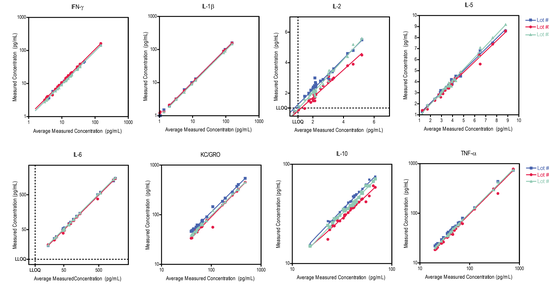
To evaluate matrix tolerance, dilution linearity was assessed by spiking recombinant protein into normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, cell culture supernatant, and urine and serially diluting the spiked samples. Different sets of samples were used for each of the 3 kit lots. Most assays showed excellent dilution linearity (within 80-120% of expected concentration) in different sample types and overall reproducibility across kit lots. IL-4 may benefit from further sample dilution, such as 8-fold or 16-fold, in serum, plasma, and urine. KC/GRO will improve with 8-fold sample dilution in urine and cell culture supernatants. The greater dilution factor may reduce the ability to detect normal levels in serum, plasma, and urine samples, but it could be viable for stimulated samples. Average recovery and standard deviations (error bars) for each assay and sample type for all 3 lots for selected matrices are depicted above. The error bars shown in the graphs represent the variability between the individual samples. We see that matrix effects are dependent on the individual samples. Some samples showed no interference. Urine samples in particular were more variable for some assays.

8 Spike Recovery

Normal mouse serum, EDTA plasma, heparin plasma, citrate plasma, cell culture media, and urine samples were spiked with calibrators at 3 different levels spanning the quantifiable range of the assay. The average recovery and the standard deviations are plotted. The figure depicts the accuracy of spiked samples in 4 of the 6 validated matrices from 3 kit lots. Independent sets of samples were used for each lot. Overall, the assays show excellent spike recovery (within 80-120% of expected concentration) and overall reproducibility across kit lots. As expected based on dilution linearity, IL-4 was typically under-recovered in serum, plasma, and urine. KC/GRO was over-recovered in urine and cell culture media.



9 Validation Samples



Lot	Statistic	IFN-γ	IL-1β	IL-2	IL-4	IL-5	IL-6	KC/GRO	IL-10	IL-12p70	TNF-α
1	r^2 Value	1.00	1.00	0.97	N/A	0.99	1.00	1.00	0.99	N/A	0.99
	Slope	0.95	1.03	1.01	N/A	0.98	0.97	1.13	1.13	N/A	1.00
2	r^2 Value	1.00	1.00	0.97	N/A	0.98	1.00	1.00	0.97	N/A	0.98
	Slope	1.09	1.02	0.90	N/A	0.96	0.98	0.93	0.83	N/A	0.98
3	r^2 Value	1.00	1.00	0.98	N/A	0.99	1.00	1.00	0.99	N/A	1.00
	Slope	0.96	0.95	1.09	N/A	1.06	1.04	0.94	1.04	N/A	1.02

To ensure reproducibility of sample recovery across lots, 5 serum, 5 EDTA plasma, 5 heparin plasma, 5 citrate plasma, and 6 stimulated plasma samples plus an unstimulated plasma control were tested across 3 lots. Correlation is reported above along with summaries of fit and slope. IL-4 and IL-12p70 did not yield enough samples in the quantifiable range to evaluate the correlation for these assays. All other assays yielded excellent correlation (all slopes within 20% of unity and most within 5% of unity).

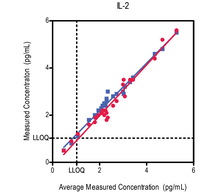
10 Specificity

To assess specificity, each assay in the panel was tested individually for each kit lot. Non-specific binding measured using individual detection antibodies was less than 0.1% for all assays in the kit. Nonspecific binding was also evaluated with additional recombinant mouse analytes (IL-13, IL-17, GM-CSF, MCP-1, MIP-3a, RANTES, TNF-RI, TNF-RII, and VEGF). Nonspecific binding from the additional mouse analytes was less than 0.2% for all assays in the kit. This level of non-specificity was verified by comparing with results from 1) running each assay with individual calibrator and individual detection antibody on the 10-spot plate, and 2) running the assays with individual calibrators and blended detection antibodies. All non-specific binding was below 0.8 %.

11 Impact of Multiplexing

To verify the absence of an impact of multiplexing, correlation for recovery of IL-2 in samples was evaluated between 10-plex and single-spot assays. Correlation was good (slopes within 5% of unity). This demonstrates that the assays used in the Proinflammatory Panel 1 (mouse) provide very similar performance as single-spot assays or as part of a multiplex.

Statistic	10-Plex	Single Spot
r^2 Value	0.98	0.98
Slope	0.96	1.04



12 Conclusion

The Proinflammatory Panel 1 (mouse) Kit, consisting of assays for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α, was validated for use with serum, EDTA plasma, heparin plasma, citrate plasma, urine, and cell culture supernatants. Three validation lots were generated (from independently produced raw material lots when possible) using established manufacturing processes. Analytical validation was performed for each kit lot, measuring sensitivity, accuracy, precision, specificity, dilution linearity, spike recovery, and recovery of samples. The development and validation data presented here demonstrate that the Proinflammatory Panel 1 (mouse) kit is a well-characterized product capable of generating highly reproducible results over multiple lots. The assays exhibit high sensitivity, specificity, reproducibility, and robustness. Multiplexing has minimal to no impact on performance. These high quality, reliable kits are excellent tools for validated studies, clinical trials, and long-term research programs.

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