W1030-04-029

Development and Characterization of Mouse Th17-Related and Cytokine Multiplex V-PLEX® Assays

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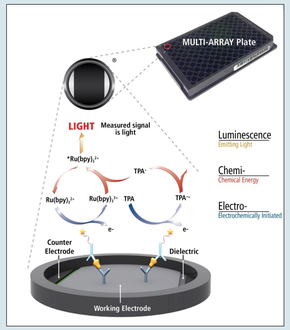
PURPOSE

Mouse models are used extensively to investigate human diseases and novel therapeutics. One of the major challenges faced when studying the growing list of biomarkers in murine models is the sample size available for testing. Sensitive assays with multiplexing capability are a solution to this problem. The Th17 subset plays a critical role in a host's defense mechanisms against various infections as well as pathogenesis of autoimmunity, inflammation, and cancer. The Th17 family of cytokines is now an addition to the expanding list of candidate biomarkers studied in relation to these indications. To address the need for sensitive and validated assays, we developed individual and multiplexed mouse Th17-related and cytokine panels on MSD's platform. The Th17 panel consists of mouse IL-17A, IL-17C, IL-17E/IL-25, IL-17F, IL-16, IL-21, IL-22, IL-23, IL-31, and MIP-3α. The cytokine panel includes mouse IL-17A/F, IL-9, IL-15, IL-30, IL-33, IP-10, MCP-1, MIP-1α, and MIP-2. These analytes can be measured with as little as 25 µL of sample, thus conserving precious sample.

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.

Challenges associated with multiplexing were solved through careful selection of antibodies and extensive diluent optimization. Analytical validation performed across multiple lots included development of optimal calibrator curves and testing for dynamic range, specificity, matrix tolerance, and limits of quantitation (LOQ). Assay robustness was evaluated at varying temperatures and sample/detector incubation times.

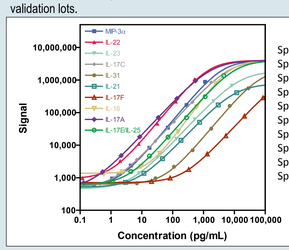
RESULTS

Assays were optimized to achieve a three-log dynamic range and a lower limit of quantitation of less than 10 pg/mL for the majority of the assays. Accuracy and precision were confirmed by testing three levels of controls across multiple lots. All three levels had concentration CVs (replicates) of less than 10% and recovery of 75-125% across runs. Matrix compatibility was demonstrated through dilution linearity and spike recovery studies in serum, plasma, urine, and cell culture media. Spiked matrices typically recovered between 75-125% of the expected value. Cross-reactivity was shown to be less than 1.0% when evaluated against thirty relevant biomarkers, with the exception of IL-17A/F.

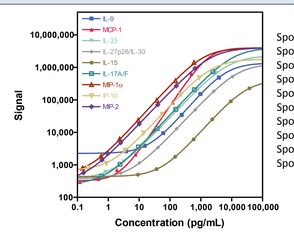
Calibration Curves and Limits of Detection

The figures below demonstrate typical calibration curves for the analytes in the V-PLEX TH17 Panel 1 (mouse) Kit (below left) and V-PLEX Cytokine Panel 1 (mouse) Kit (below right). Data include upper limit of quantitation (ULOQ), lower limit of quantitation (LLOQ), and ranges for the lower limits of detection (LLOD, n=60 runs). Calibrators were reconstituted and diluted serially (4-fold) to generate a 7-point standard curve for each panel.

The LLOD is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator). The ULOQ and LLOQ are established for each lot by measuring multiple levels near the expected LLOQ and ULOQ levels. The final LLOQ and ULOQ specifications for the product are established after assessment of all validation lots.



Spot 1: MIP-3α
Spot 2: IL-22
Spot 3: IL-23
Spot 4: IL-17C
Spot 5: IL-31
Spot 6: IL-21
Spot 7: IL-17F
Spot 8: IL-16
Spot 9: IL-17A
Spot 10: IL-17E/IL-25



Spot 1: IL-9
Spot 2: MCP-1
Spot 3: IL-33
Spot 4: IL-27p28/IL-30
Spot 5: IL-15
Spot 6: IL-17A/F
Spot 7: MIP-1α
Spot 8: IP-10
Spot 9: MIP-2
Spot 10: BSA

Calibration Curve Parameters	V-PLEX TH17 Panel 1 (mouse) Kit										V-PLEX Cytokine Panel 1 (mouse) Kit								
	MIP-3α	IL-22	IL-23	IL-17C	IL-31	IL-21	IL-17F	IL-16	IL-17A	IL-17E/IL-25	IL-9	MCP-1	IL-33	IL-27p28/IL-30	IL-15	IL-17A/F	MIP-1α	IP-10	MIP-2
ULOQ (pg/mL)	530	380	7,600	1,200	21,000	9,300	52,000	1,800	360	3,300	2,600	325	1,950	6,500	26,000	1,620	390	650	423
LLOQ (pg/mL)	3.42	1.58	4.19	0.97	22.6	12.1	320	15	0.255	4.54	21.9	4.42	1.64	5.91	43.2	1.27	0.378	2.15	0.577
LLOD Range (pg/mL)	0.191-1.01	0.064-0.281	0.367-2.91	0.117-0.766	3.51-17.5	0.820-3.95	17.1-117	1.15-12.0	0.031-0.144	0.200-2.05	0.895-13.0	0.405-1.17	0.222-0.879	0.656-4.40	8.00-39.0	0.106-0.443	0.027-0.194	0.067-3.13	0.030-0.354

Sample Testing

Normal mouse serum, plasma, and urine samples (n = 10 per matrix type) from a commercial source were diluted 4-fold and tested (below, left). To ensure the detection of all analytes in the native form, EL4 cells, J774A.1 cells, and mouse splenocytes were cultured in 90% basal media + 10% serum with antibiotics and several compounds *in vitro* to augment the synthesis of the Th17-related analytes. The supernatants were then diluted 4-fold and tested against analytes on the V-PLEX TH17 Panel 1 (mouse) and V-PLEX Cytokine Panel 1 (mouse) Kits (below, right). Most analytes were detectable within the LLOQ and ULOQ ranges thus confirming the sensitivity and utility of the panels.

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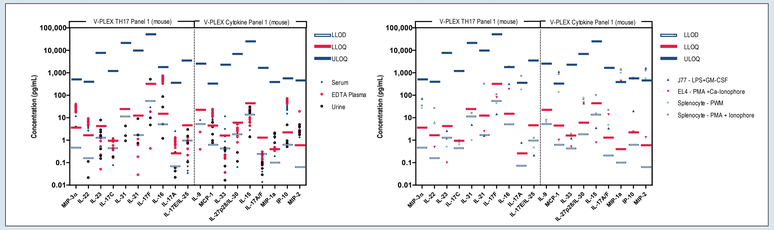


Dilution Linearity and Spike Recovery

Dilution Linearity Average % Recovery	V-PLEX TH17 Panel 1 (mouse) Kit												V-PLEX Cytokine Panel 1 (mouse) Kit											
	Serum (n=10)				EDTA Plasma (n=10)				Cell Culture Supernatant (n=6)					Serum (n=10)				EDTA Plasma (n=10)				Cell Culture Supernatant (n=6)			
Dilution Factor	4	8	16	32	4	8	16	32	4	8	16	32	Dilution Factor	4	8	16	32	4	8	16	32	4	8	16	32
MIP-3α	100	96	89	92	100	97	99	100	100	97	93	96	IL-9	100	105	104	106	100	108	110	121	100	102	102	103
IL-22	100	113	115	118	100	105	106	102	100	99	94	93	MCP-1	100	92	91	97	100	91	92	103	100	95	94	95
IL-23	100	91	99	91	100	98	97	86	100	91	88	78	IL-33	100	99	96	94	100	105	110	113	100	93	83	71
IL-17C	100	118	126	126	100	107	104	98	100	98	98	94	IL-27p28/IL-30	100	108	103	98	100	120	129	136	100	105	96	88
IL-31	100	118	136	155	100	106	110	109	100	88	81	80	IL-15	100	116	125	136	100	126	150	170	100	89	75	66
IL-21	100	102	103	98	100	100	98	93	100	105	97	96	IL-17A/F	100	99	94	88	100	104	103	105	100	96	91	79
IL-17F	100	92	92	88	100	92	86	75	100	94	86	82	MIP-1α	100	101	98	93	100	105	105	105	100	98	94	87
IL-16	100	110	108	104	100	107	106	100	100	101	96	100	IP-10	100	98	93	91	100	109	106	106	100	116	118	108
IL-17A	100	112	120	114	100	106	104	97	100	98	95	91	MIP-2	100	102	100	94	100	105	103	105	100	101	97	89
IL-17E/IL-25	100	107	109	101	100	123	121	109	100	103	100	102													

Spike Recovery Average % Recovery	V-PLEX TH17 Panel 1 (mouse)						V-PLEX Cytokine Panel 1 (mouse)					
	Serum (n=6)		EDTA Plasma (n=6)		Cell Culture Media (n=6)			Serum (n=6)		EDTA Plasma (n=6)		Cell Culture Media (n=6)	
Analytes	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Analytes	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
MIP-3α	98	89-111	80	73-85	115	99-132	IL-9	87	39-106	82	33-134	94	39-141
IL-22	82	76-90	85	77-94	114	99-130	MCP-1	94	88-104	111	103-117	125	118-131
IL-23	88	68-103	88	72-97	108	87-133	IL-33	64	48-84	65	51-72	110	98-121
IL-17C	70	54-83	77	68-85	103	82-121	IL-27p28/IL-30	61	52-76	61	39-69	98	87-106
IL-31	59	48-78	79	61-113	109	85-130	IL-15	55	48-70	62	49-69	115	103-127
IL-21	90	72-123	107	84-126	103	75-156	IL-17A/F	91	80-104	85	71-94	105	95-111
IL-17F	110	76-140	113	92-127	113	97-129	MIP-1α	96	84-108	88	69-93	106	98-117
IL-16	N/A	N/A	N/A	N/A	104	81-134	IP-10	106	98-112	76	71-82	88	75-99
IL-17A	84	74-95	84	75-100	106	92-121	MIP-2	97	88-106	86	69-97	104	97-111
Il-17E/IL-25	100	87-119	81	71-94	92	70-110							

Specificity

The V-PLEX TH17 Panel 1 (mouse) and Cytokine Panel 1 (mouse) Kits were assessed for specificity with other proinflammatory cytokines and chemokines. Thirty analytes were tested as recombinant calibrator blends: IFN-γ, IL-10, IL-12p70, IL-15, IL-16, IL-17A, IL-17A/F, IL-17C, IL17E/IL-25, IL-17F, IL-1β, IL-2, IL-21, IL-22, IL-23, IL-27p28/IL-30, IL-31, IL-33, IL-4, IL-5, IL-6, IL-9, IP-10, KC/GRO, MCP-1, MIG, MIP-1α, MIP-3α, MIP-2, TNF-α.

Non-specific binding was less than 1.0% for the assays in the panels and with other tested proteins above, except for the mouse IL-17A/F heterodimer on the IL-17A (2.64% IL-17A detect only, 5.88% blended detect) and IL-17F (7.4% IL-17F detect only, 27.8% blended detect) assays.

Dilution Linearity and Spike Recovery measurements were evaluated for different sample types throughout the quantitative range of the assays. Multiple pooled mouse serum and EDTA plasma samples were procured from a commercial source. Cell culture media was prepared using basal media +/-10% serum with antibiotics.

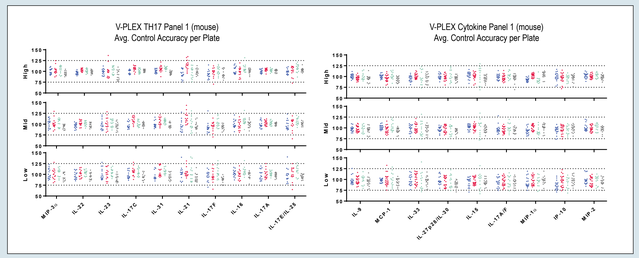
For Dilution Linearity, samples were spiked with recombinant proteins and serially diluted from 4-fold to 32-fold. For both panels, the average % recovery were within the 75-125% range for most assays. All recoveries are normalized to the recommended 4-fold dilution.

For Spike Recovery, samples were spiked with recombinant proteins at three levels (high, mid, and low) then diluted 4-fold. The average % recovery for each sample type for each assay is reported along with the % spike recovery range.

Accuracy and Precision

Quality control samples were prepared by spiking calibrator into non-mouse serum matrix at three levels (high, mid, and low) within the quantitative linear range of the assay. The controls were measured using a minimum of three replicates tested over multiple days and multiple operators for a total of 60 runs. The accuracy of control determinations fell within 25% of the expected concentration with precision of less than 20% CV.

- Accuracy is defined as the average measured control concentration for a given lot divided by the expected control concentration. The accuracy shown is an average of three replicates on a single plate. Runs were conducted using four kit lots by five different operators.



- Intra-run precision is the %CV of the control replicates within an individual run across 60 runs (four kit lots).
- Inter-run precision is the %CV from control concentrations measured on a given kit (n=15).
- Inter-lot precision (shown as %CV) is the variability of controls across 4 kit lots (total of 60 runs).

Control Precision	V-PLEX TH17 Panel 1 (mouse)			V-PLEX Cytokine Panel 1 (mouse)			
Assay	Intra-Run % CV	Inter-Run % CV	Inter-Lot % CV	Assay	Intra-Run % CV	Inter-Run % CV	Inter-Lot % CV
MIP-3α	5.6	11.6	10.1	IL-9	3.7	8.6	9.5
IL-22	3.3	6.5	6.6	MCP-1	4.4	8.7	9.0
IL-23	6.1	14.5	11.9	IL-33	3.7	11.7	11.7
IL-17C	4.2	8.0	8.2	IL-27p28/IL-30	3.7	9.1	9.2
IL-31	4.3	9.4	8.5	IL-15	5.1	11.5	12.1
IL-21	7.2	12.4	15.1	IL-17A/F	5.2	8.5	8.7
IL-17F	9.2	13.2	13.1	MIP-1α	4.2	8.0	8.9
IL-16	7.9	13.7	11.2	IP-10	4.4	10.1	10.8
IL-17A	2.8	6.6	6.2	MIP-2	3.0	7.3	7.5
IL-17E/IL-25	5.5	13.8	11.3				

CONCLUSION

Calibration curves for the V-PLEX TH17 Panel 1 (mouse IL-17A, IL-17C, IL-17E/IL-25, IL-17F, IL-16, IL-21, IL-22, IL-23, IL-31, MIP-3α) and the V-PLEX Cytokine Panel 1 (mouse IL-17A/F, IL-9, IL-15, IL-30, IL-33, IP-10, MCP-1, MIP-1α, MIP-2) demonstrated a three-log dynamic range while achieving a lower limit of quantitation of less than 10 pg/mL for the majority of the assays. Dilution linearity and spike recovery studies in serum, plasma, urine (data not shown), and cell culture media were conducted to demonstrate matrix compatibility. Cross-reactivity was shown to be less than 1.0% between assays within the panels (except in the presence of IL-17A/F) and when evaluated against thirty blood-related cytokines and biomarkers. These validated multiplexed assays provide sensitive measurement of Th17 cytokines in a variety of matrices.

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