Validation of a Multiplex Method for the Determination of Cytokine Concentrations in Rat Serum

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1 ABSTRACT

The measurement of serum cytokines can be valuable biomarkers to assess immunotoxicology. It is important to measure multiple cytokines to characterize an immune response. To conserve the amount of matrix needed for nonclinical analysis of multiple cytokines, particularly in small volume rodent samples, multiplexing can be employed. Meso Scale Discovery (MSD) offers a commercial kit for the multiplex measurement of a proinflammatory panel of cytokines in Sprague Dawley rat serum.

The purpose of this study was to validate this proinflammatory panel of cytokines for use in nonclinical analyses of rat serum samples. A 10-plex MSD kit was used to validate the measurement of the following cytokines in rat serum by electrochemilumiescence (ECL): IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, TNF- α, GM-CSF, and IFN- γ. The analytical method was validated with respect to specificity (selectivity), working calibration range, lower and upper limit of quantification (LLOQ and ULOQ), and intra- and inter-assay precision and accuracy.

All intra-assay precision and accuracy analyses were acceptable at all levels and for all cytokines. During the selectivity analysis, matrix interference was observed with serum spiked with cytokine calibrators. To address the interference, the minimum required dilution (MRD) of serum in the assay was optimized to be a 10-fold dilution. With 10% serum, selectivity at the low QC concentration level was acceptable for the following five cytokines: IL-1α, IL-5, IL-6, TNF- α, and IFN-γ. Selectivity analysis did not pass acceptance criteria for IL-1β, IL-4, IL-10, IL-13, and GM-CSF. When spiked into individual lots of rat serum, IL-1β, IL-4, IL-10, IL-13, and GM-CSF consistently under-recovered suggesting that Sprague Dawley rat serum exhibits matrix interference on the determination of IL-1β, IL-4, IL-10, IL-13, and GM-CSF concentrations in the assay. To address this during nonclinical sample analysis, relative QCs above the LLOQ were used to to trend the relative concentrations of IL-1β, IL-4, IL-10, IL-13, and GM-CSF in serum samples.

MSD offers calibrators to determine the concentration of a 10-plex panel. Although the selectivity failed acceptance criteria in this method validation, the QCs had adequate precision. The ECL method was validated with a 5-plex panel: IL-1α, IL-5, IL-6, TNF-α, and IFN-γ. The proinflammatory cytokine MSD kit is suitable for the quantitative determination of IL-1α, IL-5, IL-6, TNF- α, and IFN-γ concentrations in Sprague Dawley rat serum samples for nonclinical immunotoxicology assessments.

2 EXPERIMENTAL

This electrochemiluminescent (ECL) immunoassay is a solid phase protein assay which uses specific capture antibodies as the solid support. Each well of the 96-well microtiter plate was coated with capture antibodies in 10 distinct spots, 1 for each cytokine. Standards, QCs, and samples were added to the wells and the analytes present bound to the specific capture antibodies. All rat serum samples were diluted 10-fold in MSD Diluent 42 (diluent contains serum, blockers, and preservatives). After washing, SULFO-TAG™ conjugated secondary antibodies were added to the plate. The microtiter plare was then washed and the bound complex was detected by the addition of MSD read buffer T to the plate and subsequent excitation of the SULFO-TAG™ via an electrochemical reaction of $Ru(bpy)_3$ to generate luminescence (light), which was read using the MSD Sector 6000. The quantity of luminescence correlates with the level of cytokines present in the serum of individual samples. The cytokine concentrations were calculated using a weighted 4-parameter standard calibration curve. All standards, QCs, and samples were added to the wells in duplicate, and the mean ECL values of the duplicates are reported.

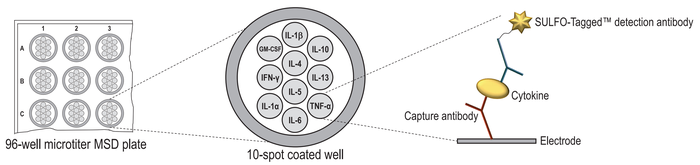

Figure 1: Diagram of the coating and assay format in a single well of a MSD microtiter plate

3 RESULTS

The mean back-calculated values for all standards were within ± 25% RE (± 30% for the LLOQ and ULOQ standards) over the ranges listed in Table 1 using a $1/y^2$ weighted 4-parameter logistic (PL) curvefit (SoftMax® Pro GxP, Version 5.0.1) that included 5 anchor points below the LLOQ.

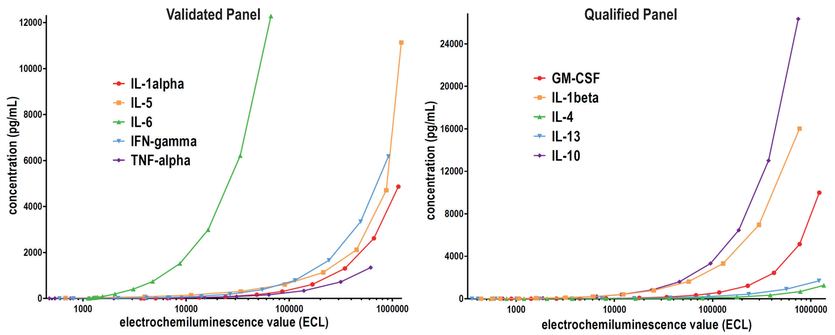

Figure 2: Representative Standard Curves of Cytokines Spiked into Assay Diluent

Selectivity was analyzed with serum lots diluted 1:10 in MSD Diluent 42. Three analytes passed acceptance criteria (IL-1α, IL-6, and TNF-α) with >80% recovering within ±25% of the theoretical concentration. For IL-5, 60% of the lots recovered within ±25% and 100% of the lots recovered within ±30%; therefore, the IL-5 selectivity analysis was considered acceptable. For IFN-γ, 40% of the lots recovered within ±25%, 60% of the lots recovered within ±30%, and 90% of the lots recovered within ±40%; therefore, the IFN-γ selectivity analysis was considered acceptable.

Table 1: Recovery (RE%) of Cytokine Calibrators Spiked Into Individual Lots of Rat Serum

Cytokine	Spiked QC (pg/mL)	Lot 1	2	3	4	5	6	7	8	9	10	Pool
		Sex F	F	F	F	F	M	M	M	M	M	N/A
Validated Panel												
IL-1α	78.13	-25	-11	-24	-27	-16	-14	-6	-17	-21	-21	-23
IL-5	145.00	-22	-19	-28	-24	-20	-27	-21	-25	-26	-27	-30
IL-6	190.63	-1	8	-1	-1	9	31	36	21	10	-4	-15
IFN-γ	98.44	-33	-27	-39	-41	-22	-22	-20	-39	-26	-24	-25
TNF-α	20.94	-9	-12	-3	-10	-4	-11	-9	-23	-15	-15	-19
Qualified Panel												
GM-CSF	156.25	-37	-31	-39	-20	-20	-42	-32	-32	-39	-37	-23
IL-1β	214.06	-62	-59	-59	-56	-52	-59	-57	-55	-54	-60	-58
IL-4	20.47	-46	-41	-48	-24	-27	-55	-40	-37	-46	-41	-30
IL-10	407.81	-41	-36	-42	-17	-23	-46	-27	-36	-36	-33	-19
IL-13	26.09	-34	-30	-29	-31	-27	-24	-28	-42	-34	-32	-28

$$RE\ (\%) = \frac{\text{back-calculated concentration} - \text{theoretical concentration}}{\text{theoretical concentration}} \times 100$$

Selectivity analysis did not pass acceptance criteria for IL-1β, IL-4, IL-10, IL-13, and GM-CSF. An investigation was conducted and determined that IL-1β, IL-4, IL-10, IL-13, and GM-CSF consistently under-recovered in this assay over the range of the standard curve in individual lots of serum. IL-1β recovery in individual lots ranged from -62% to -52% of the theoretical concentration, IL-4 recovery in individual lots ranged from -24% to -55% of the theoretical concentration, IL-10 recovery in individual lots ranged from -46% to -17% of the theoretical concentration , IL-13 recovery in individual lots ranged from -42% to -24% of the theoretical concentration, and GM-CSF recovery in individual lots ranged from -42% to -20% of the theoretical concentration.

Therefore, IL-1β, IL-4, IL-10, IL-13, and GM-CSF were not validated in this method. For sample analysis, IL-1β, IL-4, IL-10, IL-13, and GM-CSF were measured as qualified analytes with qualitative concentration measurements relative to QCs prepared in neat serum to control for matrix interference (Table 2).

3 RESULTS

For all analytes, all QC levels analyzed in assay diluent passed both intra-assay precision and relative accuracy acceptance criteria across 6 occasions with the exception of IL-1β, IL-5, and IL-6 which passed acceptance criteria on across 5 occasions and IL-5 which passed acceptance criteria on across 4 occasions. All intra-assay precision and relative accuracy analyses were acceptable at all levels and for all analytes.

Table 2: Cytokine Standard Curve Ranges in Assay Diluent and QC Concentrations in Serum

Cytokine	Assay Range (pg/mL)	LLOQ %RE	LLOQ %CV	QC1 %RE	QC1 %CV	QC2 %RE	QC2 %CV	QC3 %RE	QC3 %CV	ULOQ %RE	ULOQ %CV
Validated panel											
IL-1α	39.06 - 5000	-9	15	-6	11	-3	8	-3	8	-6	10
IL-5	72.5 - 9280	1	13	3	12	-1	8	3	12	26	19
IL-6	95.31 - 12200	4	23	-4	13	-3	7	-2	6	2	6
IFN-γ	49.22 - 6300	-3	9	-4	8	-1	10	6	13	1	13
TNF-α	10.47 - 1340	3	10	-5	9	-3	6	4	9	3	11
Qualified panel											
GM-CSF	78.13 - 10000	4	11	1	9	-4	6	-1	7	3	13
IL-1β	107.03 - 13700	10	13	-1	10	-5	6	10	13	9	14
IL-4	10.23 - 1310	3	14	1	13	6	14	4	11	-7	15
IL-10	203.91 - 26100	-3	14	-7	12	-7	8	2	8	5	12
IL-13	13.05 - 1670	3	7	-9	7	-2	4	5	5	0	3

4 CONCLUSION

The selectivity analysis during the method validation resulted in consistent recovery ranges to those reported by MSD (Table 3). IL-1β, IL-4, IL-10, IL-13, and GM-CSF all under recovered and did not meet method validation percent recovery acceptance criteria when spiked into individual lots of rat serum.

MSD offers calibrators to determine the concentration of a 10-plex panel. Although the selectivity failed acceptance criteria in this method validation, the QCs had adequate precision. The ECL method was validated with a 5-plex panel: IL-1α, IL-5, IL-6, TNF-α, and IFN-γ. The proinflammatory cytokine MSD kit is suitable for the quantitative determination of IL-1α, IL-5, IL-6, TNF- α, and IFN-γ concentrations in Sprague Dawley rat serum samples for nonclinical immunotoxicology assessments.

Table 3: Recovery Ranges of Serum Lots Spiked with Cytokine Calibrators.

Cytokine	Selectivity Analysis (n=10) % Recovery Range	Selectivity Analysis (n=10) Average % Recovery	MSD Kit Analysis (n=5)[b] % Recovery Range	MSD Kit Analysis (n=5)[b] Average % Recovery
Validated panel				
IL-1α	73-94	82	N/A[a]	N/A[a]
IL-5	72-81	76	68-101	84
IL-6	96-136	111	92-126	109
IFN-γ	59-80	71	50-81	61
TNF-α	77-97	89	48-94	76
Qualified panel				
GM-CSF	58-80	67	N/A[a]	N/A[a]
IL-1β	38-48	43	36-50	43
IL-4	45-76	60	69-104	85
IL-10	54-83	66	39-78	54
IL-13	58-76	69	46-107	77

[a] GM-CSF and IL-1α were ordered as custom calibrators and were not included in the standard MSD rat multiplex kit.
[b] Obtained from the MSD Proinflammatory Panel 2 [rat] Kit Product Insert (Catalog No. K15059).

For sample analysis, QC will be prepared in neat serum and standards will be prepared in kit diluent. All serum samples and QCs will be diluted to 10% serum in the assay. An LLOQ quality control sample for the qualified cytokines (when diluted in the assay) will be prepared at a concentration greater than the lowest standard. This low concentration QC will be used to account for the under-recovery of the qualified panel at the low end of the assay range.

5 ACKNOWLEDGEMENTS

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