Rat Cytokines and Acute Phase Protein Multiplexes for Preclinical Toxicology

This poster presents multiplex panels of biomarkers for systemic inflammation, vasculitis and tissue injury. These panels have been designed for use in preclinical studies: they measure rat analytes and are formatted for use in a regulated laboratory. Our Acute Phase Protein Panel 1 measures α_2-macroglobulin (A2M) and α_1-acid glycoprotein (AGP), two acute phase proteins that are induced in systemic vascular injury and chronic inflammation. Assays that measure these markers for preclinical toxicology have been challenging since rats provide limited sample and reagents for assays have been scarce. We also present multiplex panels for Rat Cytokines; these panels are relevant to inflammation and tissue damage. These panels have advantages that are typical of assays from Meso Scale Discovery (MSD): greater sensitivity, reduced sample volume, a greater dynamic range (both endogenous and elevated levels can be measured at a single dilution factor) and improved throughput. Kits containing these assays are now available for purchase from MSD.

Description of Markers

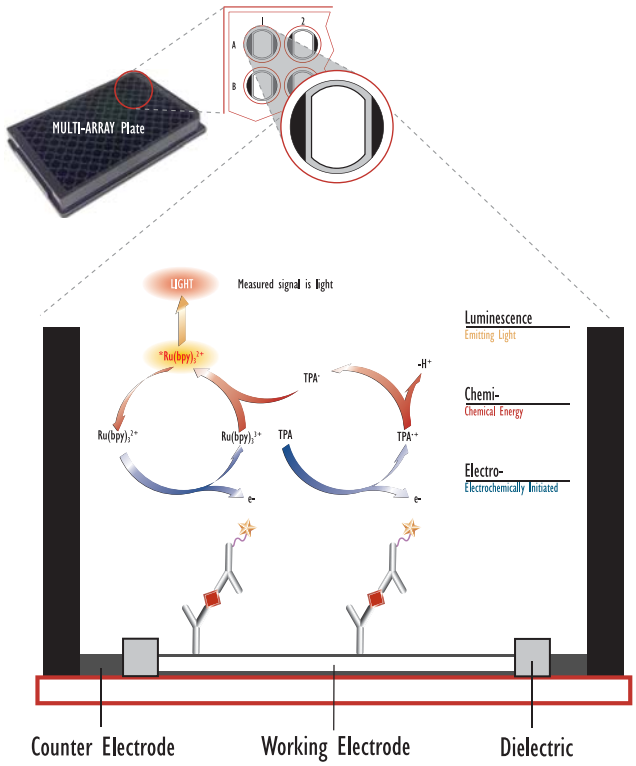
Rat α_2-macroglobulin (A2M) is a serum glycoprotein produced by hepatocytes. It is induced in acute and chronic inflammatory injury. Rat α_1-acid glycoprotein (AGP) is involved with acute phase inflammatory response; its specific function is unknown, but it is involved promotion of growth of fibroblasts. There is no homolog to A2M in human plasma. Both A2M and AGP are abundant in rat serum: levels elevate after an inflammatory response and can remain elevated for several days. AGP may be indicative of liver hypertrophy as well as local and general inflammation.

Cytokines and chemokines are often classified by their origin and function (assays for rat cytokines currently available from MSD are shown in **bold**):

- Th1 signature cytokines: **IFN-γ** and IL-12 (IL-12 is only available for mouse & human species); also Type 1 cytokines include **IL-2** and **TNF-α**.
- Th2 signature cytokines: **IL-4** and **IL-13**; also Type 2 cytokines include **IL-5**, **IL-6**, and **IL-10**.
- Pro-inflammatory cytokines/chemokine: **TNF-α**, IL-1β, and IL-12, **MCP-1**, and GM-CSF; **IL-1α** is expressed by murine inflammatory macrophages and human keratinocytes.
- Anti-inflammatory cytokines: **IL-4**, **IL-13**, and **IL-10**. Other anti-inflammatory mediators include IFN-α & G-CSF.
- Pleiotropic cytokine involved in acute inflammatory reaction: **IL-6**
- B-cell secreted chemokines: **CXCL1 (KC/GRO)**, **MIP-3α**

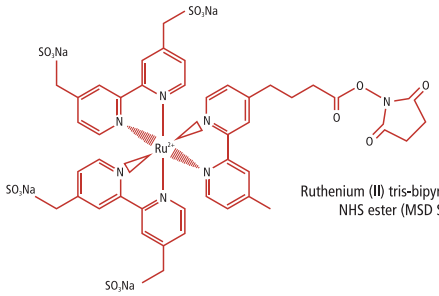
The MSD® Platform

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 Features:

- Minimal background signals and high signal to background ratios - the stimulation mechanism (electricity) is decoupled from the signal (light)
- Proximity - only labels bound near the electrode surface are detected, enabling non-washed assays
- Flexibility - labels are stable, non-radioactive, and are conveniently conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple excitation cycles of each label enhance light levels and improve sensitivity

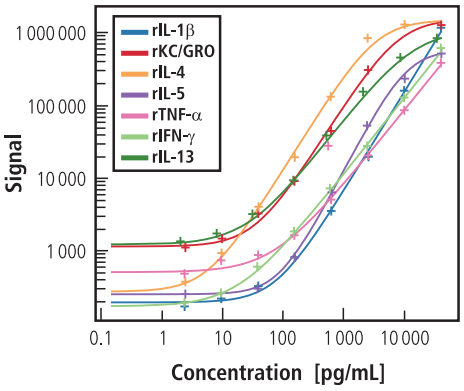


Ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester (MSD SULFO-TAG™ label)

Ultrasensitive Rat 7-Plex Cytokine Panels

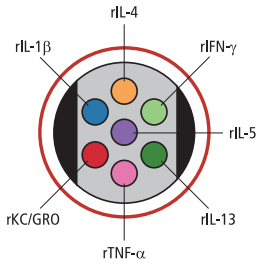
MSD's Ultrasensitive Rat 7-Plex Cytokine Panel measures seven rat cytokines simultaneously from a single sample (serum or plasma). Typical standard curves for this panel are shown in the figure below. The lower limit of detection (LLOD) for each analyte was determined by calculating 2.5 standard deviations above the average background (no analyte). LLOD's range from 1.6 pg/mL to 40.5 pg/mL (see table). The linear dynamic range is very large: these assays remain linear beyond 20000 pg/mL. The typical %CV for the standard curves and samples were less than 10%. We also show another multiplex panel of rat cytokines: this assay was prepared as a custom panel. Standard curves and LLODs are presented in the figures and table below. The protocol shown below is typical for MSD rat cytokine assays; samples are run without dilution.

Rat Demonstration Panel



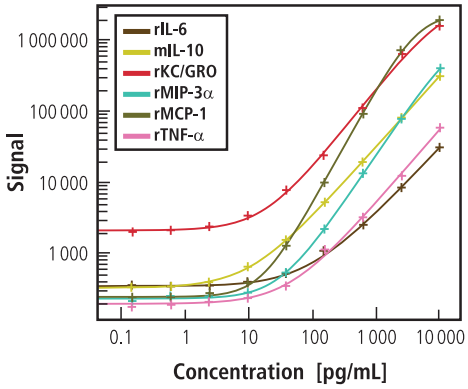
Concentration (pg/mL)	rIL-1β	rKC/GRO	rIL-4	rIL-5	rTNF-α	rIFN-γ	rIL-13
0	193	1088	264	266	468	178	1192
2.4	177	1126	382	264	487	189	1367
9.8	226	1516	965	264	772	270	1709
39.1	336	3365	4083	316	901	605	3250
156.3	835	9230	19870	837	1683	1819	9217
625	3609	45708	133195	7005	5135	7110	39309
2500	19661	313727	843987	54070	20755	27335	154037
10000	163618	1158641	1280540	239962	85368	124362	463971
40000	1174268	1246818	1301215	556837	392376	602236	837085

	rIL-1β	rKC/GRO	rIL-4	rIL-5	rTNF-α	rIFN-γ	rIL-13
LLOD (pg/mL)	25	9	2	41	10	5	6



	Limits of Detection Across Lots (pg/mL)						
	rIL-1β	rKC/GRO	rIL-4	rIL-5	rTNF-α	rIFN-γ	rIL-13
Median	20	3.0	1.1	32	11	6.5	4.4
Max	50	9.0	1.9	128	59	20	6.0
Min	11	1.3	0.3	20	4.0	5.2	3.2
N of Lots	10	6	7	5	9	7	5

Custom Panel



Concentration (pg/mL)	rIL-6	rIL-10	rKC/GRO	rMIP-3α	rMCP-1	rTNF-α
0	356	314	2101	243	246	202
2.4	348	389	2432	246	270	208
10	373	629	3432	277	384	232
39	455	1533	7836	529	1282	341
156	1066	5288	24641	2230	10022	1082
625	2540	19378	113559	13424	94312	3188
2500	8350	81233	668328	78591	739763	12415
10000	31837	324046	1654324	404738	1936650	60518

	rIL-6	rIL-10	rKC/GRO	rMIP-3α	rMCP-1	rTNF-α
LLOD (pg/mL)	25	2	5	14	7	14

Ultrasensitive Rat 7-Plex Cytokine Panels

Cross-Reactivity of Analytes

Cross-reactivity of the assays within the same well was measured using a single analyte at 10000 or 40000 pg/mL. We chose these high values to yield a high specific signal and to provide a stringent test for cross reactivity. The signals measured for the other analytes were used to calculate cross-reactivity. The % cross-reactivity was less than 0.5% for all analytes and typically undetectable at levels below 0.2%.

	rIL-1β	rKC/GRO	rIL-4	rIL-5	rTNF-α	rIFN-γ	rIL-13
rIL-1β spot	100%	0.12%	0.12%	0.09%	0.04%	0.08%	0.02%
rKC/GRO spot	0.01%	100%	-0.12%	-0.16%	-0.45%	-0.09%	-0.16%
rIL-4 spot	0.20%	0.08%	100%	0.15%	0.08%	0.19%	0.04%
rIL-5 spot	0.08%	0.11%	0.08%	100%	0.06%	0.12%	0.04%
rTNF-α spot	0.42%	0.17%	0.24%	0.37%	100%	0.11%	0.35%
rIFN-γ spot	0.08%	0.06%	0.10%	0.15%	0.24%	100%	0.06%
rIL-13 spot	-0.50%	-0.02%	-0.51%	-0.39%	-0.75%	0.03%	100%

Protocol:

1 Add 25 µL of MSD RSC Assay Diluent, incubate 30 min at RT.

2 Add 25 µL of standard/sample, incubate 2 hours at RT.

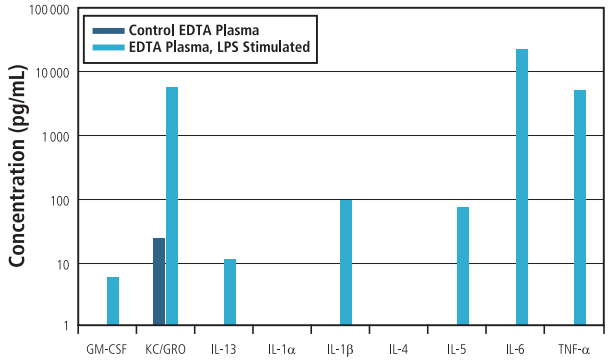
3 Wash with PBS-T. Add 25 µL of Detection Antibody, incubate 2 hours at RT.

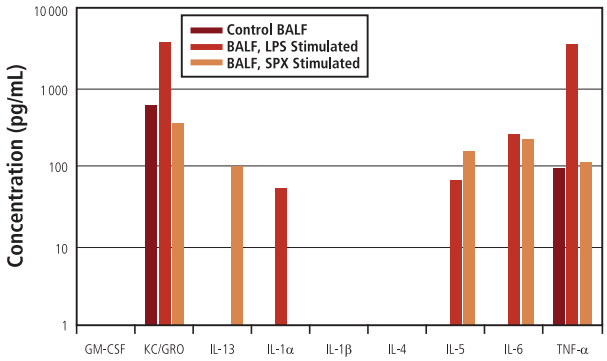
4 Wash with PBS-T. Add 150 µL of Read Buffer T, read.

Cytokine Responses in Plasma and BALF

Rat EDTA plasma samples and bronchoalveolar lavage fluids (BALF) samples were tested using another multiplexed cytokine panel. Representative data from the study are shown in the table and graphs below. Plasma from rats treated with systemic lipopolysaccaride (LPS) showed higher levels of KC/GRO, IL-6, IL-1β and TNF-α when compared to plasma from control rats. Cytokine levels also increased in BALF from rats treated with either LPS or sephadex (SPX): LPS treatment induced more than 100-fold increases in cytokine and chemokine levels. The extended dynamic range of the MSD assays is particularly advantageous under these conditions. The MSD assay can measure cytokines in all samples at a single dilution factor, despite 100- to 1000-fold differences in cytokine abundance between controls and treated samples.

	Concentration (pg/mL)								
	GM-CSF	KC/GRO	IL-13	IL-1α	IL-1β	IL-4	IL-5	IL-6	TNF-α
Control EDTA Plasma	< 4.6	22	< 10.4	< 10.8	< 29	< 6.2	< 50	< 145	< 35
EDTA Plasma, LPS Stimulated	5	5579	11	< 10.8	89	< 6.2	75	23225	4883
Control BALF	< 4.6	603	< 10.4	< 10.8	< 29	< 6.2	< 50	< 145	95
BALF, LPS Stimulated	< 4.6	3571	< 10.4	51	< 29	< 6.2	64	244	3511
BALF, SPX Stimulated	< 4.6	348	101	< 10.8	< 29	< 6.2	157	211	112



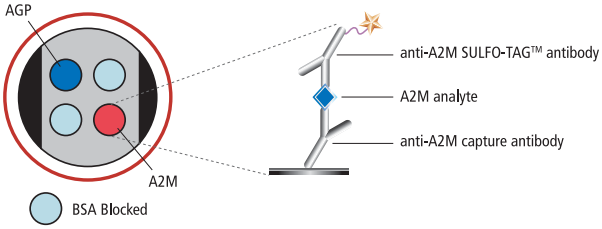


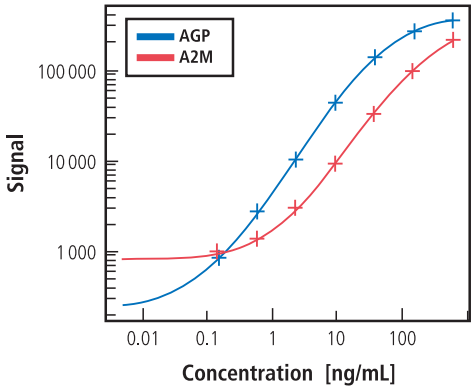
Acute Phase Protein Panel I (rat): A2M and AGP

The MSD Acute Phase Protein Panel 1 measures A2M and AGP in serum and plasma. We qualified this panel according to typical practices for pre-clinical biomarkers. The qualification procedure involved multi-day controls, establishment of limits of quantitation, spike recovery, dilutional linearity, and measurement of control and treated samples. The assay showed good separation between controls and treated samples. These markers are abundant (µg's/mL) and require a 20000 fold dilution of the serum and plasma samples. They are usually measured separately from cytokines (since cytokines are present at much lower levels and are usually measured in neat samples).

Standard Curve

The MSD Acute Phase Protein Panel 1 is quantitative over a 1000-fold range. Representative standard curves from a typical run are shown below. The lower limit of detection (LLOD) for each analyte was determined by calculating 2.5 standard deviations above the average background (no analyte). LLOD for AGP was 0.012 ng/mL; the LLOD for A2M was 0.036 ng/mL. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were assigned following a multi-day study. We assigned the LLOQ (or ULOQ) as the lowest (or highest) concentration where the %CV of the calculated concentration was less than 20% and the percent recovery of the concentration was between 80% and 120%.





Concentration (ng/mL)	AGP		A2M	
	Mean	% CV	Mean	% CV
0	263	3.9	815	1.8
0.15	876	1.6	964	5.3
0.59	2757	3.9	1368	3.4
2.34	10279	2.0	2989	2.4
9.38	43117	3.4	8946	3.0
37.5	138099	2.5	31833	3.0
150	266110	3.2	96516	4.0
600	349199	1.5	210212	1.5

Protocol:

1 Add 25 µL of Antibody Diluent, incubate 30 min at RT.

2 Add 25 µL of standard and/or sample, incubate 2 hours at RT.

3 Wash with PBS-T. Add 25 µL of Detection Antibody, incubate 2 hours at RT.

4 Wash with PBS-T. Add 150 µL of Read Buffer T, read.

	AGP (ng/mL)	A2M (ng/mL)
LLOD	0.012	0.036
LLOQ	0.146	1.00
ULOQ	150	400

Precision: Multi-Day Study

High, mid, and low controls were defined that are rat serum samples diluted 10000-fold into assay diluent. Samples were chosen that span a large proportion of the dynamic range for each assay. These controls were run in triplicate on each of 9 plates run across 3 days of testing. The concentration values are corrected for the 10000-fold dilution.

				Intra-plate			Inter-plate
	Control	Plates	Avg. Conc. (ng/mL)	Average % CV	Max % CV	Min % CV	% CV
AGP	High	9	42706	5.3	8.7	2.8	9.9
	Mid	9	8427	4.2	7.3	2.6	10.1
	Low	9	408	3.4	4.8	2.7	7.1
A2M	High	9	215203	6.2	13.1	1.8	8.6
	Mid	9	20468	2.4	3.2	0.3	6.3
	Low	9	1870	3.1	4.9	0.7	4.0

Acute Phase Protein Panel I (rat): A2M and AGP

Spike Recovery

Heparin plasma and EDTA plasma were spiked with the standards at multiple concentrations throughout the range of the assay. Samples were spiked with the A2M and AGP recombinant proteins after they were diluted 10000-fold. The spike recoveries were between 80% and 120%.

% Recovery = measured / expected * 100

	AGP		A2M	
	Spike Level (ng/mL)	% Recovery	Spike Level (ng/mL)	% Recovery
EDTA Plasma	18.3	98	223.4	110
	9.2	104	108.8	107
	4.4	104	49.8	106
Heparin Plasma	102.5	97	102.5	92
	25.6	110	25.6	103
	6.4	108	6.4	105

Dilutional Linearity

Several serum samples with endogenous levels throughout the range of the assays were diluted with the MSD Antibody Diluent from 10000 fold to 80000 fold.

% Recovery = (measured * dilution factor) / expected * 100

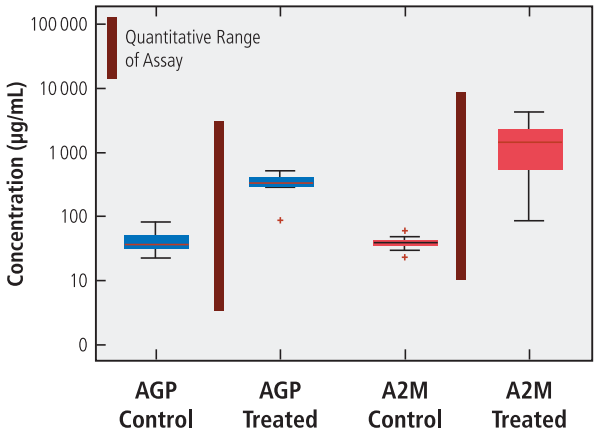
	AGP				A2M			
	Dilution	Adjusted Conc. (ng/mL)	% CV	% Recovery	Dilution	Adjusted Conc. (ng/mL)	% CV	% Recovery
EDTA Plasma 1	10000	4964074	4.1		10000	388695	1.7	
	20000	4755761	2.2	96	20000	398111	4.4	102
	40000	4020536	1.1	84	40000	353202	1.2	89
	80000	4024289	2.8	100	80000	354821	3.7	100
EDTA Plasma 2	10000	433803	2.3		10000	197042	6.5	
	20000	401829	1.6	93	20000	212536	10.5	108
	40000	353441	1.7	88	40000	191266	4.5	90
	80000	352253	3.3	100	80000	198975	3.2	104
Serum	10000	20726	5.7		10000	18273	3.0	
	20000	18961	8.2	91	20000	17593	1.7	96
	40000	18085	4.3	95	40000	16998	2.1	97
	80000	< LLOQ	15.7	108	80000	16789	3.1	99

Samples

EDTA plasma samples from 15 control animals and 9 animals treated with agents to induce an inflammatory response were run at a 20000-fold dilution. The table shows the corrected concentrations for controls and treated samples. The graph shows the range of measurements for both sets of animals along with the quantitative range of the assay. Several treatments produced more than 100-fold induction of AGP and a 10-20 fold induction in A2M. The large dynamic range of the assay eliminated the need for re-testing samples at multiple dilution factors.

		Endogenous Analyte Levels in Samples	
		AGP (µg/mL)	A2M (µg/mL)
Control EDTA Plasma	Mean	39	41
	Median	38	36
	Range	23.1 - 59.7	21.6 - 79.7
Treated EDTA Plasma	Mean	1645	332
	Median	1404	329
	Range	84 - 4142	86.9 - 502.1

Detected level was above LLOQ for all analytes in all samples. Average CVs for measured samples was less than 10%.



Conclusions

Inflammatory cells transiently secrete cytokines into the bloodstream in response to injury or inflammation; these cytokines regulate the acute-phase protein response. MSD has developed high performance, multiplex assays to measure biomarkers of inflammation, vasculitis and tissue injury. The combination of multiplexing, wide dynamic range and increased throughput enables studies that measure many analytes from small pre-clinical samples. MSD has released the Acute Phase Protein Panel I as a fully qualified kit. Many multiplex panels of cytokines are also available without full qualification at this time.