Qualification of Kidney Injury Markers for Preclinical Studies

Traditional clinical markers for kidney injury such as BUN and serum creatinine are not sensitive enough to detect subtle kidney damage and often do not correlate to damage measured by histopathology. This poster describes multiplex panels of traditional and novel biomarkers for kidney injury that can overcome these shortcomings. Our Kidney Injury Panel 1 (rat) includes Albumin, TIM-1 (a.k.a KIM-1 or HAVCR), NGAL (a.k.a.Lipocalin-2), and Osteopontin. Albumin is a common marker for kidney damage; TIM-1, NGAL and Osteopontin are emerging biomarkers. We also present individual assays for TIM-1 and for Clusterin, another novel biomarker for kidney injury. 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

Albumin is an abundant serum protein that acts as a transport protein for hemin and fatty acids. Albumin is produced in the liver and secreted in the bloodstream. Damage to the kidney can lead to albuminuria, secretion of albumin into the urine.

T cell immunoglobulin and mucin domain containing molecule-1 (TIM-1/KIM-1/HAVCR) is a type 1 transmembrane glycoprotein found on CD4+ T cells and renal proximal tubule epithelial cells. The extracellular domain of TIM-1 is made of an immunoglobulin-like domain topping a long mucin-like domain, suggesting a possible role in cell adhesion. TIM-1 is released upon certain types of acute kidney injury and can be measured in urine, serum, or plasma.

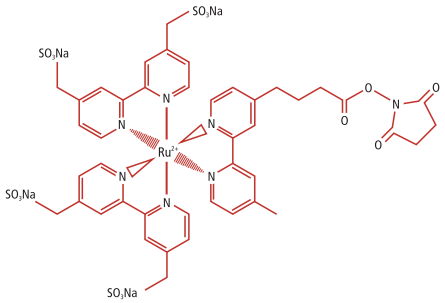
Lipocalin-2 (a.k.a. Neutrophil Gelatinase-Associated Lipocalin (NGAL)) is a 25 kDa protein that acts as a transport protein, carrying small hydrophobic molecules such as steroid hormones, vitamins, and metabolic products. Lipocalin-2 is expressed in most tissues and is induced in epithelial cells upon inflammation. In the kidney, Lipocalin-2 may be implicated in both progress and protection from renal injury.

Osteopontin (OPN) is a glycoprotein that is involved in bone metabolism, immune regulation, cell survival, and tumor progression. OPN is mostly expressed in bone, kidney, and epithelial tissues.

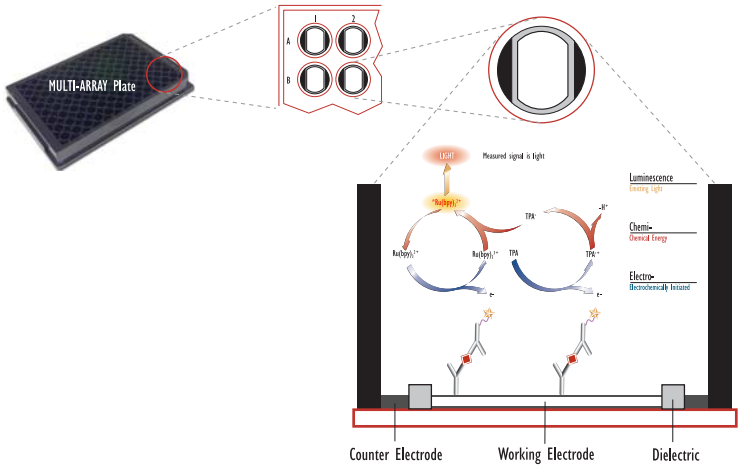
Clusterin is a glycoprotein that is found in most mammalian tissues. The localized over-expression of clusterin at sites of tissue damage or stress implicates clusterin as a molecular chaperone displaying cytoprotective characteristics. In addition, the marked induction of clusterin in several renal disease states suggest that clusterin is a biochemical marker of kidney damage and disease.

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.



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

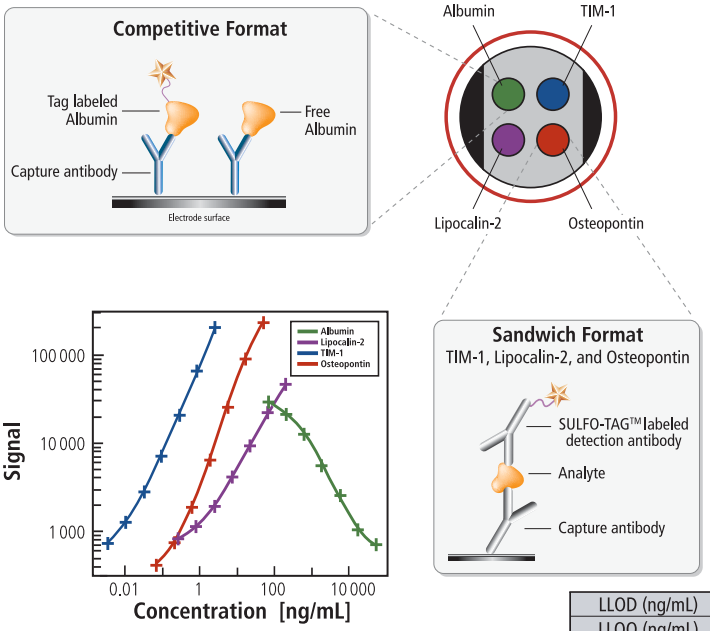


Electrochemiluminescence Features:

- 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

Kidney Injury Panel 1 (rat): Albumin, TIM-1, Lipocalin-2, Osteopontin

Our Kidney Injury Panel 1 (rat) measures Albumin, TIM-1, Lipocalin-2 (NGAL), and Osteopontin (OPN) in urine samples. Albumin is very abundant (10's of µg/mL); we measure it by competitive immunoassay to enable multiplexing with lower-abundance biomarkers. The other analytes are measured by sandwich immunoassays. 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. Representative standard curves from a typical run are shown below. The lower limit of detection (LLOD) for TIM-1, NGAL, and OPN was determined by calculating 2.5 standard deviations above the average background (no analytes); the LLOD for albumin was set at 80% of the maximum signal. 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 calculated concentration of the standard was between 80% and 120%.



Albumin		
Concentration (ng/mL)	Average Counts	% CV
0	32482	16
69	29260	4.7
206	21374	1.2
617	12544	5.2
1852	5489	15
5556	2542	6.2
16667	1048	3.7
50000	707	37

Lipocalin-2		
Concentration (ng/mL)	Average Counts	% CV
0	629	1.8
0.274	833	2.5
0.823	1147	4.5
2.47	1938	4.6
7.41	4109	3.3
22.2	9661	3.0
66.7	21445	5.5
200	45046	8.6

TIM-1		
Concentration (ng/mL)	Average Counts	% CV
0	414	5.7
0.003	729	1.8
0.010	1311	1.8
0.031	2877	3.7
0.093	7157	2.5
0.278	21183	4.2
0.833	65911	2.3
2.50	205155	2.0

Osteopontin		
Concentration (ng/mL)	Average Counts	% CV
0	352	11
0.069	431	8.1
0.206	765	2.7
0.617	1940	2.5
1.85	6832	1.5
5.56	26485	5.8
16.7	92889	1.9
50	233443	5.4

	Albumin
LLOD (ng/mL)	108
LLOQ (ng/mL)	781
Upper limit (ng/mL)	28481

	TIM-1	Lipocalin-2	Osteopontin
LLOD (ng/mL)	0.0013	0.250	0.066
LLOQ (ng/mL)	0.02	1.56	0.39
ULOQ (ng/mL)	1.88	150	37.5

Precision: Multi-Day Study

A multi-day, multi-plate study over 9 plates was performed to show reproducibility. In addition to the standard curves, control samples (high, mid, and low) were measured on each plate. Each sample was run in triplicate. The average intra-plate %CV and inter-plate %CV of the concentrations are shown below.

	Control	Plates	Concentration (ng/mL)	Average interplate % CV (n=3 plates)	Interday % CV (n=9 plates)
Albumin	High	9	9628	13.7	15.9
	Mid	9	4845	14.1	15.7
	Low	9	1593	17.5	20.5
TIM-1	High	9	0.80	7.4	8.3
	Mid	9	0.15	10.5	13.4
	Low	9	0.04	10.3	11.8
Lipocalin-2	High	9	58.4	10.3	10.3
	Mid	9	10.8	14.2	16.0
	Low	9	2.71	12.7	15.7
Osteopontin	High	9	3.99	11.0	12.1
	Mid	9	2.16	9.8	13.6
	Low	9	2.00	8.5	11.5

Protocol:

1 Add 150 µL Blocking Solution, incubate 1 hour at RT.

2 Wash with PBS-T. Add 50 µL of sample or standard premixed with Albumin tracer. Incubate 2 hours.

3 Wash with PBS-T. Add 25 µL of detection antibody blend. Incubate 2 hours.

4 Wash with PBS-T. Add150 µL Read Buffer and read.

Dilutional Linearity

	Albumin (pooled control urine)			TIM-1 (urine from treated rat)			Lipocalin-2 (urine from treated rat)			Osteopontin (pooled control urine)		
Sample Dilution Factor	Dilution Corrected Conc. (ng/mL)	Conc. CV	% Recovery	Dilution Corrected Conc. (ng/mL)	Conc. CV	% Recovery	Dilution Corrected Conc. (ng/mL)	Conc. CV	% Recovery	Dilution Corrected Conc. (ng/mL)	Conc. CV	% Recovery
5	25957	23.3		0.74	2.5		73.7	12.7		20.2	8.5	
10	30928	4.1	119	0.74	17.9	100	94.9	0.9	129	41.5	3.0	206
20	30482	32.4	99	0.86	17.6	116	126	2.2	133	67.9	2.6	163

Urine samples were tested at 5, 10, and 20-fold dilution to measure linearity.

Spike Recovery

Rat urine samples were spiked with the calibrators at multiple values throughout the range of the assay. The spiked samples were tested at a 10-fold dilution into the assay diluent. The recombinant osteopontin may be bound to proteins in the urine, making it under-recover. Diluting the sample did not show linear dilution of the osteopontin. At a single dilution, the %CV of the samples are very consistent; therefore, with the large dynamic range of the assay, we can measure both control samples and treated samples without diluting the samples differently.

Albumin Spike Level (ng/mL)	Concentration (ng/mL)	Concentration CV	% Recovery
7812	10412	11.0	94
1953	4846	1.1	94
0	3225		

TIM-1 Spike Level (ng/mL)	Concentration (ng/mL)	Concentration CV	% Recovery
6.25	5.913	0.7	94
1.56	1.584	2.0	99
0.39	0.430	2.4	102
0.098	0.131	7.8	102
0.024	0.058	0.4	105
0	0.031	5.0	

Lipocalin-2 Spike Level (ng/mL)	Concentration (ng/mL)	Concentration CV	% Recovery
62.5	60.6	8.7	81
15.6	25.8	2.8	91
0	12.7	5.3	

Osteopontin Spike Level (ng/mL)	Concentration (ng/mL)	Concentration CV	% Recovery
4.88	4.6	3.4	68
1.2	2.3	2.8	72
0	1.9	0.8	

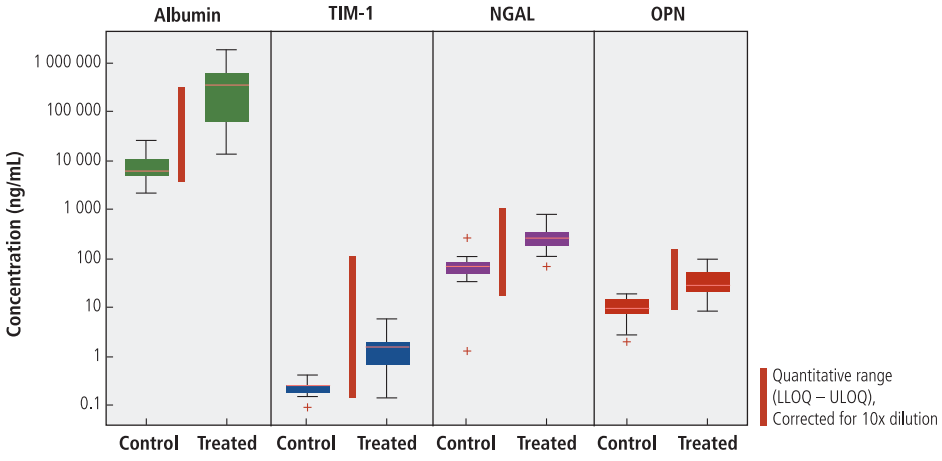
Kidney Injury Panel 1 (rat): Albumin, TIM-1, Lipocalin-2, Osteopontin

Samples

Rat urine samples were assayed at 10-fold dilution on the Kidney Injury Panel 1. The "treated" samples tested were from rats exposed to known nephrotoxicants prior to sample collection. High Tox designated samples that assayed above the ULOQ were assayed again at 40-fold dilution. For all of the analytes on the panel, significant correspondence between histo-pathology score and abundance is observed. Concentrations in green were below the LLOQ for the analyte designated.

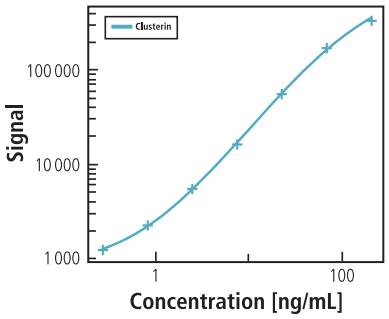
Animal #	Designation	Histopathology Score	Albumin Conc., (ng/mL)	TIM-1 Conc., (ng/mL)	Lipocalin-2 Conc., (ng/mL)	Osteopontin Conc., (ng/mL)
1	control	0	6380	0.26	79.4	2.0
2	control	0	4676	0.22	81.8	10.6
3	control	0	5639	0.15	54.0	6.8
4	control	0	2172	0.09	50.9	5.7
5	control	0	7981	0.21	80.2	9.5
6	control	0	13063	0.27	32.8	10.4
7	control	0	4099	0.15	68.2	9.3
8	control	0	25787	0.24	43.6	8.7
9	control	0	6010	0.25	68.6	17.7
10	control	0	10219	0.16	48.1	14.7
11	mild tox	1	24359	0.14	68.9	8.5
12	mild tox	1	582857	1.66	216	20.1
13	mild tox	1	347477	2.43	185	18.7
14	mild tox	2	63056	1.99	185	25.8
15	mild tox	2	190213	0.84	110	18.1
16	mild tox	3	593019	1.34	184	28.1
17	mild tox	2	409532	1.56	264	47.6
18	mild tox	2	1375229	1.92	242	59.7
19	mild tox	2	438888	2.00	325	53.4
20	high tox	3	1398916	1.91	334	32.5

Animal #	Designation	Histopathology Score	Albumin Conc., (ng/mL)	TIM-1 Conc., (ng/mL)	Lipocalin-2 Conc., (ng/mL)	Osteopontin Conc., (ng/mL)
21	high tox	3	814377	0.64	345	41.4
22	high tox	3	1822741	1.29	308	39.5
23	high tox	3	141078	0.37	134	17.0
24	high tox	3	122467	0.39	144	24.0
25	high tox	4	352434	0.45	297	24.7
26	high tox	3	20037	3.38	689	40.8
27	high tox	3	13009	1.18	309	75.7
28	high tox	3	25402	5.11	493	27.8
29	high tox	3	71925	5.76	800	67.7
30	high tox	3	42825	4.00	665	96.0
31	control	0	10563	0.26	261	2.7
32	control	0	4724	0.25	51.8	12.8
33	control	0	26265	0.18	109	7.1
34	control	0	5412	0.27	73.2	17.4
35	control	0	3501	0.19	96.5	8.3
36	control	0	5178	0.25	1.3	7.9
37	control	0	5138	0.34	56.7	12.5
38	control	0	10322	0.41	70.1	19.0
39	control	0	12564	0.26	41.9	15.9



Rat Clusterin Assay

The MSD rat clusterin assay has a quantitative range of 0.82 - 150 ng/mL, and is designed for use with rat urine samples. 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 urine samples from treated rats. The lower limit of detection (LLOD) was determined by calculating 2.5 standard deviations above the average background (no analyte). 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%. Representative standard curve from a typical run is shown below.



Clusterin		
Conc. (ng/mL)	Mean	% CV
0	660	6.2
0.27	1217	5.9
0.82	2232	5.4
2.47	5470	2.8
7.41	16359	4.1
22.2	55620	4.9
66.7	172202	3.6
200	338824	3.4

	Clusterin (ng/mL)
LLOD	0.017
LLOQ	0.82
ULOQ	150

Protocol:

1. Add 150 µL Blocking Solution, incubate 1 hour at RT.
2. Wash with PBS-T. Add 25 µL Assay Diluent then 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.

Dilutional Linearity

		Clusterin			
Sample	Dilution Factor	Signal	Adjusted Conc. (ng/mL)	% CV	% Recovery
Sample 1	5	18015	43.6	3.22	
	10	11362	55.9	2.63	128
	20	6521	64.4	0.48	115
	40	3524	67.0	1.98	104
	80	1946	64.5	3.33	96.2
Sample 2	5	13037	31.9	1.54	
	10	7412	36.6	3.31	115
	20	4250	41.2	1.39	112
	40	2414	42.8	3.95	104
	80	1485	42.8	13.6	100
Sample 3	5	5238	12.9	4.69	
	10	3055	14.2	2.54	111
	20	1791	14.3	2.22	101
	40	1207	14.6	5.42	102
	80	946	15.8	6.46	108
Sample 4	5	39582	103	2.98	
	10	27103	141	2.35	138
	20	17649	187	3.48	132
	40	10067	216	1.74	116
	80	5551	240	1.17	111
Sample 5	5	29196	70.5	2.46	
	10	19232	94.4	1.84	134
	20	12634	126	2.04	133
	40	6922	139	0.64	111
	80	4012	158	2.22	113

Serial dilutions of a several rat urine samples were tested to assess linearity. Measurements below the assay LLOQ are shown in grey.

Spike Recovery

Rat urine samples were spiked with clusterin standard at multiple levels throughout the range of the assay. The spiked samples were tested at either 5-fold or 10-fold dilution into the assay diluent. Control sample 1 is a urine sample from an individual rat, and control sample 2 is a pooled urine sample.

		Clusterin			
Sample	Spike level, ng/mL	Expected Conc., ng/mL.	Measured Conc., ng/mL.	% CV	% Recovery
Control sample1, 5-fold dilution	124	125	161	2.49	129
	14.1	14.9	17.1	2.30	115
	0.57	1.38	1.54	5.34	112
Control sample 2, 5-fold dilution	124	125	118	4.50	94.4
	14.2	14.9	13.9	6.10	92.9
	0.67	1.48	1.44	5.86	97.1
Control sample 2, 10-fold dilution	124	125	119	2.68	95.2
	14.6	15.0	13.9	3.11	92.7
	1.08	1.51	1.44	0.64	95.5

Rat Clusterin Assay

Precision: Multi-Day Study

High, mid, and low controls were made by spiking recombinant protein into stock calibrator diluent. The controls were run in quadruplicate on each of 10 plates run across three days. The controls were run at a 10-fold dilution.

				Intra-plate			Inter-plate
	Control	Plates	Ave. Conc. (ng/mL)	Average % CV	Max % CV	Min % CV	% CV
Clusterin	High	10	139	7.27	16.8	2.16	10.4
	Mid	10	17.5	4.30	9.13	0.95	6.06
	Low	10	1.90	4.80	10.7	0.89	6.57

Samples

Urine samples from control and treated rats were tested for clusterin. Treated animals were dosed with nephrotoxicants prior to sample collection. Samples were assayed at 5X dilution; some samples were retested at higher dilution. Values highlighted in green were less than the dilution-adjusted LLOQ of 4.1 ng/mL.

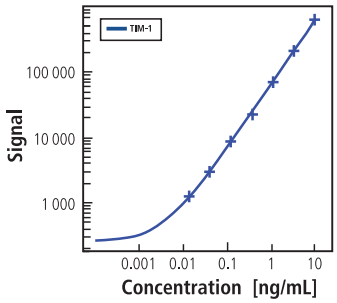
Clusterin		
Sample #	Type	Dilution Corrected Conc., (ng/mL)
1	control	8.4
2	control	1.8
3	control	4.2
4	control	8.3
5	control	9.3
6	control	4.0
7	control	6.6
8	control	7.3
9	control	7.3
10	control	3.9
11	control	2.5

Clusterin		
Sample #	Type	Dilution Corrected Conc., (ng/mL)
12	control	12.3
13	control	0.8
14	control	1.0
15	control	1.6
16	control	9.8
17	control	11.0
18	control	6.5
19	treated	9.0
20	treated	469
21	treated	270
22	treated	591

Clusterin		
Sample #	Type	Dilution Corrected Conc., (ng/mL)
23	treated	464
24	treated	1428
27	treated	111
28	treated	31.0
29	treated	83.0
30	treated	84.9
34	treated	356
35	treated	143
36	treated	213
37	treated	230
38	treated	231

Rat TIM-1/KIM-1/HAVCR Assay

MSD now offers a single-analyte assay for TIM-1. We qualified this assay 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 lower limit of detection (LLOD = 0.001 ng/mL) was determined by calculating 2.5 standard deviations above the average background (no analyte). 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%. This assay was quantitative over a 500- to 1000-fold range: we assigned an LLOQ of 0.02 ng/mL and a ULOQ of 10 ng/mL. Representative standard curve from a typical run is shown below.



TIM-1		
Conc. (ng/mL)	Mean	% CV
0	259	5.1
0.014	1237	2.7
0.041	3036	3.1
0.123	8727	3.3
0.370	22906	5.1
1.11	70952	6.0
3.33	212959	2.6
10	641896	3.3

	TIM-1 (ng/mL)
LLOD	0.001
LLOQ	0.02
ULOQ	10

Protocol:

1. Add 150 µL Blocking Solution, incubate 1 hour at RT.
2. Wash with PBS-T. Add 25 µL MRSC Antibody Diluent then 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.

Precision: Multi-Day Study

High, mid, and low controls were made by spiking recombinant protein into pooled rat urine. The low control was the endogenous pooled rat urine. The controls were run in triplicate on each of 9 plates run across three days. The controls were run at a 10-fold dilution.

				Intra-plate			Inter-plate
	Control	Plates	Ave. Conc. (ng/mL)	Average % CV	Max % CV	Min % CV	% CV
TIM-1	High	9	0.821	6.8	15.8	1.3	14.0
	Mid	9	0.298	3.8	7.7	1.5	13.6
	Low	9	0.118	4.3	8.5	2.2	14.5

Rat TIM-1/KIM-1/HAVCR Assay

Spike Recovery

Rat urine samples were spiked with the standards at multiple levels throughout the range of the assay. The spiked samples were tested at a 5-fold dilution into the assay diluent.

TIM-1 Spike Level (ng/mL)	Expected Concentration (ng/mL)	Measured Concentration (ng/mL)	% CV	% Recovery
10	10.05	8.98	7.2	89
2.5	2.55	2.37	5.9	93
0.625	0.675	0.67	5.0	99
0.312	0.362	0.39	0.7	107
0.156	0.156	0.23	1.3	112
0	0.05	0.05	3.7	

Dilutional Linearity

Serial dilutions of a rat urine sample were tested to assess linearity. At 80-fold dilution, the concentration was below the assay LLOQ.

	TIM-1			
Dilution	Signal	Adjusted Conc. (ng/mL)	% CV	% Recovery
1	9190	0.495	9.7	
5	2752	0.741	2.5	149
10	1443	0.739	17.9	100
20	913	0.858	17.6	116
40	540	0.814	7.9	95
80	368	0.766	20.7	94

Conclusions

MSD has developed high performance, multiplex assays to measure biomarkers of kidney injury. Composed of both traditional and emerging biomarkers, these panels can indentify kidney injury and may help stratify different types of kidney damage. The combination of multiplexing, wide dynamic range, and increased throughput enables studies that measure many analytes from small pre-clinical samples. The analytes presented here have been studied to verify a positive correlation between the results of the MSD assays and results from traditional histopathology. MSD has released certain panels as preconfigured, fully qualified kits. Other panels are available on a custom basis without full qualification.

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