Development and Validation of Multiplexed Human Kidney Biomarkers

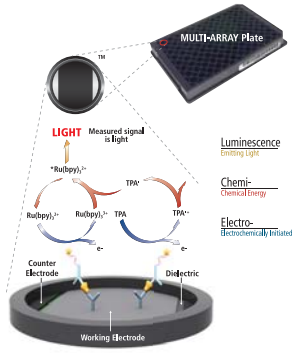
Measurement of protein markers as indicators of drug-induced kidney toxicity shows promise in improving drug safety and accelerating development timelines.[1] Fifteen exploratory biomarkers of kidney toxicity were reviewed, and assays were assembled based on the relative abundance of these biomarkers in human urine.

- Kidney Injury Panel 3 (KIP3) measures the levels of low abundance biomarkers: αGST, calbindin, clusterin, KIM-1, osteoactivin, TFF3 and VEGF.
- Kidney Injury Panel 4 (KIP4) measures the levels of mid abundance biomarkers: πGST and RBP4
- Kidney Injury Panel 5 (KIP5) measures the levels high abundance biomarkers: albumin, Beta-2 microglobulin (B2M), cystatin C, epidermal growth factor (EGF), NGAL, osteopontin (OPN) and uromodulin (UMOD).

MESO SCALE DISCOVERY® developed these multiplexed panels following fit-for-purpose principles,[2] FDA Bioanalytical Method Validation guidance and CLSI documents. Data from KIP3 and KIP5 are presented in this poster. MSD multiplex panels use simple protocols and low sample volumes (less than 10 µL). The panels were validated for sensitivity, specificity, dilution linearity, spike recovery, precision, accuracy, robustness and sample handling. Preliminary sample data showed the levels of kidney injury biomarkers, including KIM-1, B2M, cystatin C, NGAL and UMOD, were significantly elevated in urine and serum samples from patients with kidney disease.

The MSD® Platform

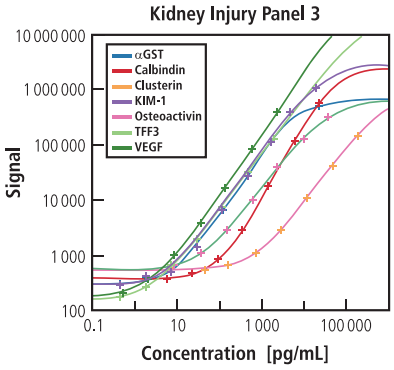
MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.

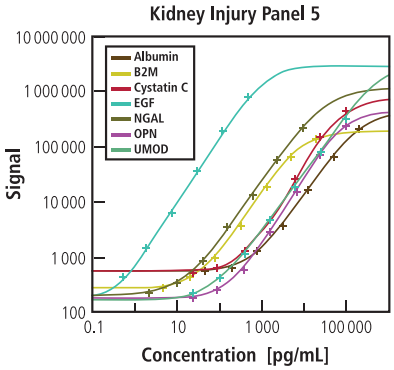


Electrochemiluminescence Features

- Minimal non-specific backgrounds and strong signal responses to analyte yield high signal to background ratios
- The stimulation mechanism (electricity) is decoupled from the response (light signal)
- Proximity assay - only labels bound near the electrode surface are excited, enabling non-washed assays
- Flexibility - labels are stable, are non-radioactive, and directly conjugated to biological molecules
- Emission at ~620 nm - eliminating problems with color quenching
- Signal amplification - multiple rounds of excitation and emission of each label enhance light levels and improve sensitivity
- Carbon electrode surface has 10X greater binding capacity than polystyrene well
- Surface coatings can be customized

Kidney Injury Panels Standard Curve





Protocol

1. Add 150 µL blocking solution. Incubate for 30 min at room temperature (RT).
2. Wash with PBS-T. Add 50 µL of standard or diluted sample (MSD recommends 1:10 and 1:500 dilution for KIP3 and KIP5, respectively. Thus, actual sample volume needed is much less than 10 µL.). Incubate for 2 hours at RT.
3. Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 2 hours at RT.
4. Wash with PBS-T. Add 150 µL of Read Buffer T; read on MSD SECTOR® Imager.

The above standard curves illustrate typical data from assay development and validation. 8-point standard curve was prepared by 4-fold serial dilution. A weighted 4PL nonlinear regression model was used for standard curve-fitting analysis. Results show a wide dynamic range (3–4 logs) that provides the ability to measure treated and untreated samples in the same dilution.

Assay Sensitivity

	Kidney Injury Panel 3						
	αGST	Calbindin	Clusterin	KIM-1	Osteoactivin	TFF3	VEGF
LLOD (pg/mL)	1.76	11.0	54.4	0.784	5.57	1.14	0.670
LLOQ (pg/mL)	8.00	100	80.0	20.0	40.0	4.00	2.50
ULOQ (pg/mL)	1 800	22 500	180 000	18 000	36 000	1 800	2 250

	Kidney Injury Panel 5						
	Albumin	B2M	Cystatin C	EGF	NGAL	OPN	UMOD
LLOD (pg/mL)	107	5.72	26.5	0.116	1.75	150	15.0
LLOQ (pg/mL)	600	60.0	120	0.750	200	200	60.0
ULOQ (pg/mL)	190 000	17 000	30 000	475	8 500	95 000	95 000

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the average value of multiple blanks (N=24).

Multi-plate, multi-day runs (N=6) were conducted to establish the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) with acceptable precision (≤25%).

Specificity

	Blended Calibrator and Single Detection, % Specificity						
Spot	αGST	Calbindin	Clusterin	KIM-1	Osteoactivin	TFF3	VEGF
αGST	**100.0**	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Calbindin	0.1	**100.0**	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Clusterin	< 0.1	< 0.1	**100.0**	< 0.1	< 0.1	< 0.1	< 0.1
KIM-1	< 0.1	< 0.1	< 0.1	**100.0**	< 0.1	< 0.1	< 0.1
Osteoactivin	< 0.1	< 0.1	< 0.1	< 0.1	**100.0**	< 0.1	< 0.1
TFF3	< 0.1	0.3	< 0.1	< 0.1	< 0.1	**100.0**	< 0.1
VEGF	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	**100.0**

	Blended Detection and Single Calibrator, % Specificity						
Spot	αGST	Calbindin	Clusterin	KIM-1	Osteoactivin	TFF3	VEGF
αGST	**100.0**	< 0.1	0.2	< 0.1	< 0.1	< 0.1	< 0.1
Calbindin	0.1	**100.0**	0.3	0.1	< 0.1	< 0.1	< 0.1
Clusterin	0.0	< 0.1	**100.0**	0.1	< 0.1	< 0.1	< 0.1
KIM-1	< 0.1	< 0.1	0.4	**100.0**	< 0.1	< 0.1	< 0.1
Osteoactivin	0.1	< 0.1	0.3	0.1	**100.0**	< 0.1	0.1
TFF3	0.1	< 0.1	0.1	< 0.1	< 0.1	**100.0**	< 0.1
VEGF	0.1	< 0.1	0.1	< 0.1	< 0.1	< 0.1	**100.0**

Assay selectivity and specificity was tested using a blended calibrator with single detection antibody or blended detection antibodies with a single calibrator. No significant cross reactivity (<0.5%) was observed for KIP3 or KIP5.

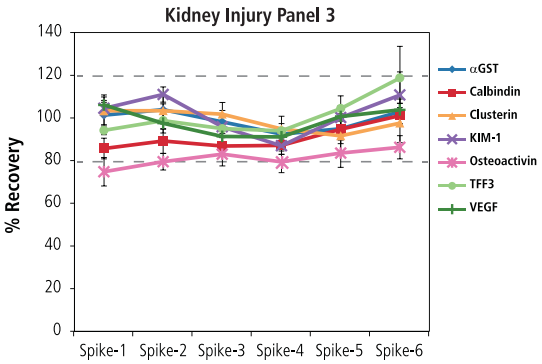
Precision: Multi-Day Study

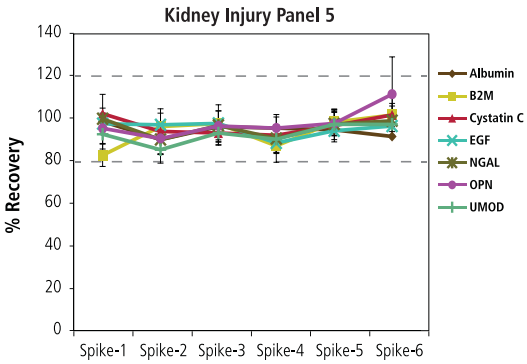
	Kidney Injury Panel 3				
	Control	Plates	Average Conc. (pg/mL)	Average Intra-plate %CV	Inter-plate %CV
αGST	High	6	1 019	5.3	19.0
	Mid	6	119	2.5	19.0
	Low	6	11.0	5.5	18.2
Calbindin	High	6	10 302	4.9	4.5
	Mid	6	1 394	4.5	4.5
	Low	6	172	3.5	3.2
Clusterin	High	6	28 946	5.4	5.2
	Mid	6	5 782	12.4	11.4
	Low	6	771	8.7	16.2
KIM-1	High	6	16 656	8.0	7.9
	Mid	6	2 360	3.6	3.3
	Low	6	107	2.5	3.9
Osteoactivin	High	6	46 191	10.3	9.2
	Mid	6	2 687	5.6	6.0
	Low	6	230	5.0	5.6
TFF3	High	6	1 019	6.7	6.6
	Mid	6	173	5.2	5.6
	Low	6	29.1	4.1	4.2
VEGF	High	6	1 045	3.5	4.2
	Mid	6	119	3.6	5.0
	Low	6	10.4	6.0	5.6

	Kidney Injury Panel 5				
	Control	Plates	Average Conc. (pg/mL)	Average Intra-plate %CV	Inter-plate %CV
Albumin	High	11	82 714	7.0	10.4
	Mid	11	15 052	6.8	13.9
	Low	11	2 688	3.4	9.5
B2M	High	11	3 728	6.5	13.8
	Mid	11	563	3.4	8.6
	Low	11	119	4.7	10.1
Cystatin C	High	11	32 816	6.2	23.6
	Mid	11	4 364	4.3	13.9
	Low	11	823	4.1	14.7
EGF	High	11	134	8.2	14.1
	Mid	11	39.7	7.3	14.5
	Low	11	2.40	6.1	12.2
NGAL	High	11	3 105	5.2	9.7
	Mid	11	2 328	3.4	10.9
	Low	11	40.5	6.2	16.6
OPN	High	11	9 380	6.5	18.4
	Mid	11	3 157	7.6	16.0
	Low	11	349.2	7.1	19.9
UMOD	High	11	28 985	4.9	14.3
	Mid	11	5 660	4.1	13.4
	Low	11	1 254	3.1	10.5

High, mid and low controls were prepared by spiking calibrator into normal human urine samples based on endogenous levels. These urine matrix-based controls were tested over multiple days across multiple plates; the panels demonstrated good precision.

Spike Recovery





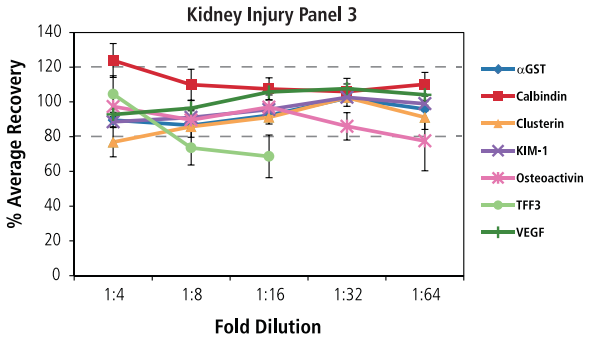
	Spike Concentration (pg/mL)					
	Spike-1	Spike-2	Spike-3	Spike-4	Spike-5	Spike-6
αGST	1 600	400	100	25.0	6.25	1.56
Calbindin	20 000	5 000	1 250	313	78.1	19.5
Clusterin	160 000	40 000	10 000	2 500	625	156
KIM-1	32 000	8 000	2 000	500	125	31.3
Osteoactivin	16 000	4 000	1 000	250	62.5	15.6
TFF3	1 600	400	100	25.0	6.25	1.56
VEGF	2 000	500	125	31.3	7.81	1.95

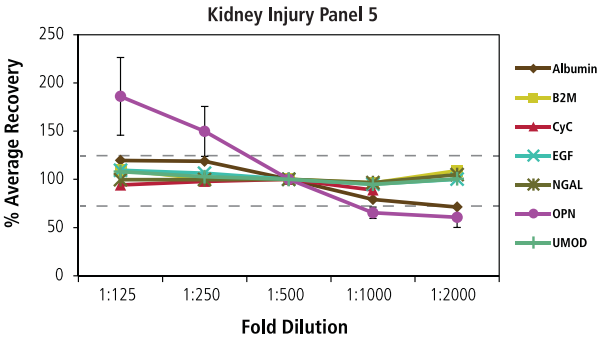
	Spike Concentration (pg/mL)					
	Spike-1	Spike-2	Spike-3	Spike-4	Spike-5	Spike-6
Albumin	160 000	40 000	10 000	2 500	625	156
B2M	16 000	4 000	1 000	250	62.5	15.6
Cystatin C	32 000	8 000	2 000	500	125	31.3
EGF	400	100	25.0	6.25	1.56	0.391
NGAL	8 000	2 000	500	125	31.3	7.81
OPN	80 000	20 000	5 000	1 250	313	78.1
UMOD	80 000	20 000	5 000	1 250	313	78.1

Eight, individual, human normal urine samples were diluted 20-fold (KIP3) or 500-fold (KIP5) then spiked with calibrators at multiple levels.

The average recovery and standard deviation was calculated from these sample sets. The error bar is the standard deviation calculated from these samples. Spike recovery for most of the samples was between 80-120%.

Dilution Linearity

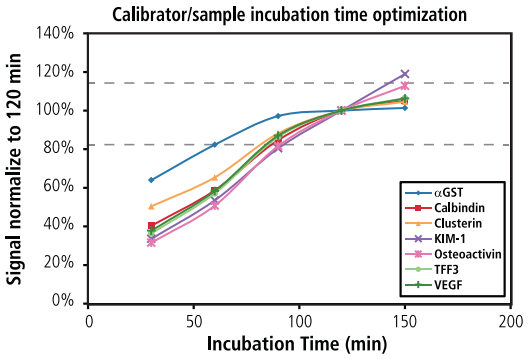


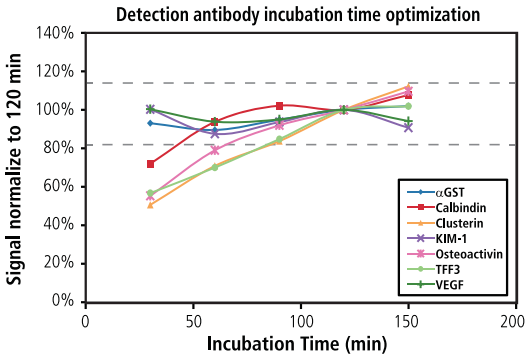


To assess linearity, a dilution series of multiple human normal urine samples (N=8 for KIP3, N=10 for KIP5) was tested for recovery. The % average recovery was calculated from these samples. The error bar is the standard deviation calculated from these samples. Data showed good dilutional linearity for all of the analytes, except OPN and TFF3. OPN is known to bind to calcium crystals in urine and cause measurement inaccuracy at low dilutions. TFF3 endogenous levels are low; it might require less dilution.

Robustness

Protocol Time

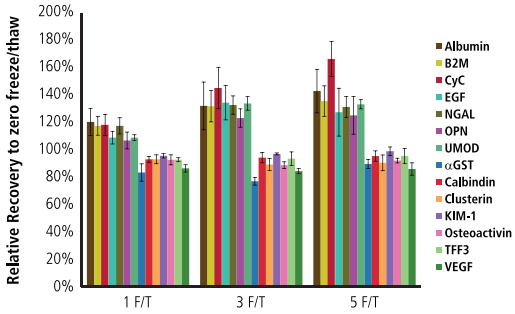




A protocol time tolerance study was conducted with a series of time points. 2 hours incubation time for calibrator or detection antibody was optimal.

The data demonstrated that assay incubation time can vary by 30 min in either direction with signals remaining within 15%.

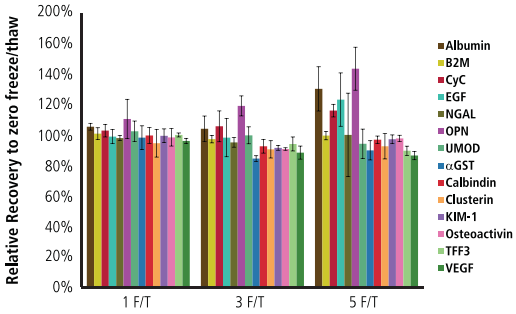
Calibrator Freeze/Thaw Stability



KIP3 and KIP5 calibrators were tested for 5 freeze/thaw cycles. Data showed some analytes, including albumin, B2M, cystatin C and NGAL, are not stable for multiple freeze/thaw cycles.

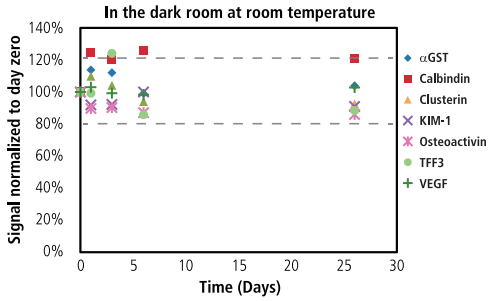
MSD does not recommend multiple freeze/thaw cycles for calibrators.

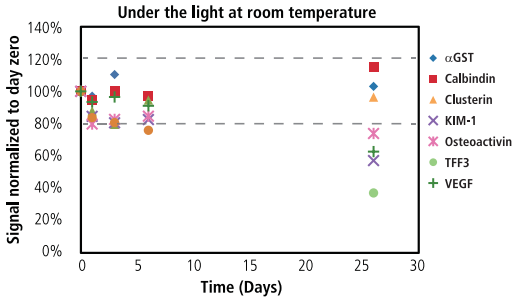
Control Freeze/Thaw Stability



Controls at different levels were made by spiking calibrator into urine. These controls were tested for 5 freeze/thaw cycles. Data showed some controls were not stable at 5 freeze/thaw cycles. Based on these data, MSD does not recommend multiple freeze/thaw cycles for controls.

Detection Antibody Accelerated Stability

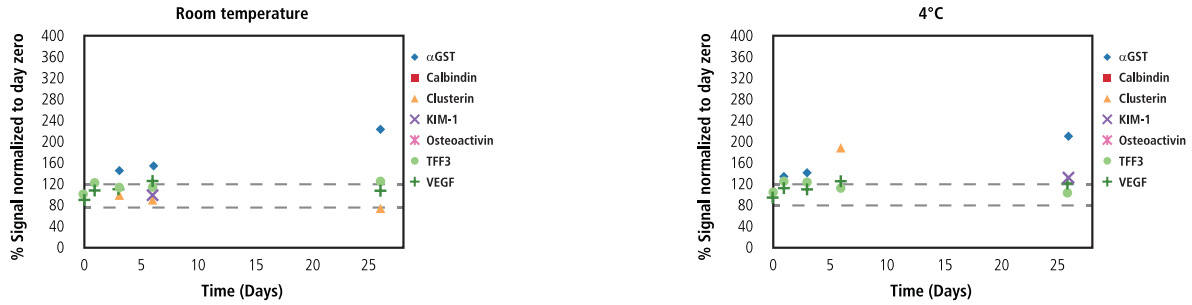




Detection antibodies were stored with or without light exposure at room temperature up to 26 days. Data from KIP3 showed some detection antibodies are sensitive to light, but all have good long term stability in the dark. MSD recommends storing detection antibody in the dark at 4°C.

Robustness

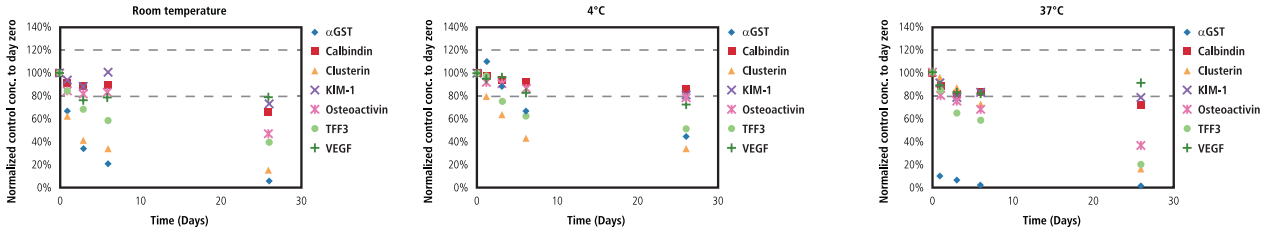
Calibrator Accelerated Stability



Blended calibrator from KIP3 was stored under different conditions (4°C or room temperature) up to 26 days. Data showed αGST and clusterin are sensitive to these conditions.

MSD recommends storing calibrator at ≤-70°C.

Controls Accelerated Stability

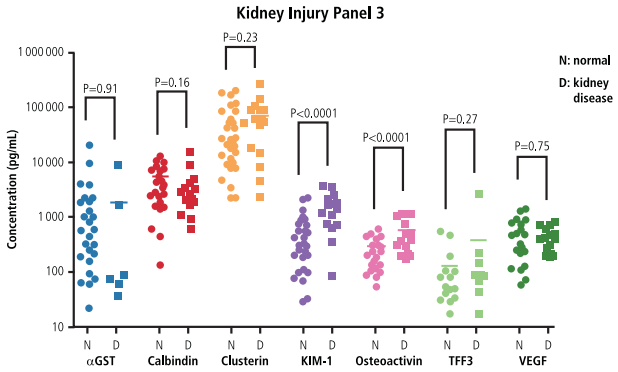


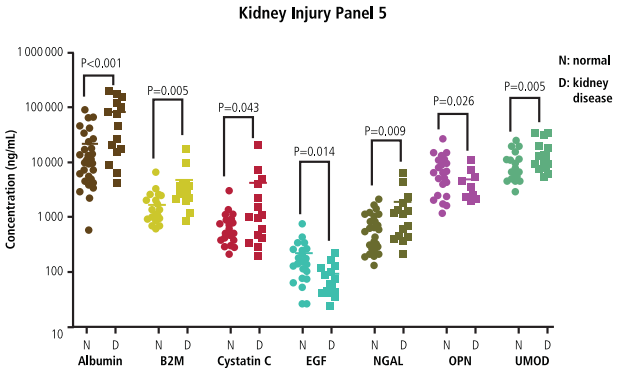
KIP3 controls were made with spiking calibrator into urine matrices. These controls were stored under different conditions (4°C, room temperature or 37°C) up to 26 days. Data showed most of the analytes are sensitive to these conditions, suggesting urine samples should be processed and frozen quickly.

MSD recommends storing controls at ≤-70°C.

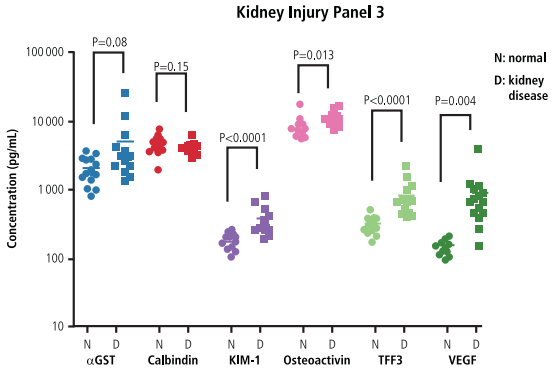
Samples

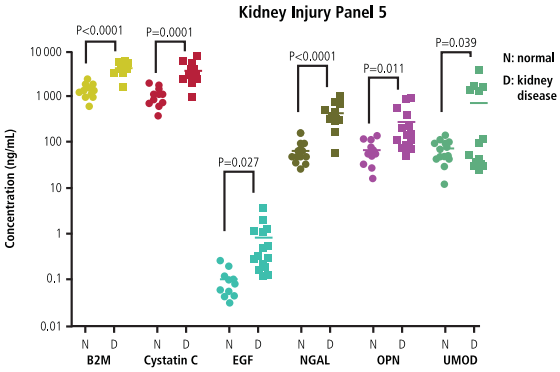
Urine Samples





Serum Samples





Human urine and serum samples from normal and kidney disease patients were obtained from Bioreclamation. Kidney disease samples are from a variety of patients associated with kidney damage or injury. Clinical information associated with these samples was not available.

Urine samples: n=35 normal; n=15 kidney disease

Serum samples: n=15 normal; n=15 kidney disease

For most analytes, the panels detected significant differences between normal samples and kidney disease samples.

Albumin and clusterin are known to be highly abundant in serum. They require higher dilution compared to urine.

Conclusions

MSD kidney injury panels (human) can measure important kidney biomarkers and have been validated with bioanalytical methods for accuracy, precision, sensitivity, reproducibility and stability.

MSD assays offer simple protocols with minimal sample volume requirements. The Kidney Injury Panels (human) perform well in both urine and serum matrices and can measure native levels of kidney biomarkers in normal and disease samples simultaneously without multiple dilutions.

Kidney biomarkers, including KIM-1, osteoactivin, cystatin C, B2M , NGAL and UMOD, were significantly elevated in both urine and serum kidney disease samples. These panels can be useful tools for researchers studying kidney toxicity or damage.

Custom assay kits are available upon request.

References

[1] Dieterle F, et al. Renal biomarker qualification submission: a dialog between the FDA-EMEA and Predictive Safety Testing Consortium. Nat Biotechnol. 2010

[2] Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, Keller S, Weinryb I, Green M, Duan L, Rogers JA, Millham R, O'Brien PJ, Sailstad J, Khan M, Ray C, Wagner JA. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006, 23(2):312-28., 28(5):455-62.

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