

Bio-Plex[®] suspension array system

tech note 6400

Profiling of Human, Canine, and Rat Urine Samples Using Bio-Plex Pro[™] RBM Kidney Toxicity Assays

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Introduction

Acute kidney injury (AKI) is a serious medical condition caused by a variety of bodily insults, including trauma from an accident, blood loss from a medical procedure, or exposure to nephrotoxic drugs or chemicals. In fact, drug exposure is estimated to cause up to 25% of all cases of AKI in critically ill patients (Bonventre et al. 2010). From a pharmaceutical industry standpoint, drug-induced toxicity is a serious issue, sidelining 30% of therapeutics overall — from preclinical lead compounds to marketed drugs (Bonventre et al. 2010).

The current clinical criterion for the diagnosis of AKI relies on glomerular filtration rate (GFR), blood urea nitrogen (BUN), and serum creatinine (SCr), which are neither sensitive nor specific (Slocum et al. 2012). Traditional tests of this nature employ late biomarkers that are detectable only days or weeks after kidney damage has occurred. Researchers and clinicians are looking for biomarkers that are sensitive, specific, and early indicators of AKI. In addition, biomarker level monitoring should be cost effective and permit rapid reporting of results.

In collaboration with Myriad RBM and the Predictive Safety Testing Consortium (PSTC), we have developed the Bio-Plex Pro[™] RBM kidney toxicity assay panels (Table 1). Based on Luminex xMAP technology, these immunoassays enable robust measurement of multiple kidney toxicity/injury biomarkers in human, canine, and rat urine samples. The multiplex format allows researchers to detect key proteins that may be upregulated within hours of kidney damage, providing valuable information throughout the drug development process — from lead optimization to preclinical and clinical protocol decision making.

Table 1. Bio-Plex Pro RBM multispecies kidney toxicity assays.

	Assays											
	Albumin	Clusterin	B2M	Calbindin	Cystatin C	GST-π	KIM-1	IL-18	MCP-1	NGAL	Osteopontin	TFF3
Human Kidney Toxicity												
Panel 1		•		•		•	•	•	•			
Panel 2	•		•		•					•	•	•
Canine Kidney Toxicity												
Panel 1		•					•		•	•		
Albumin	•											
Rat Kidney Toxicity												
Panel 1		•					•	•	•		•	
Panel 2			•	•	•					•		
Albumin	•											

Method

The Bio-Plex Pro RBM kidney toxicity assays employ a standard sandwich enzyme immunoassay method using a 96-well plate format. The capture antibody-coupled beads are allowed to react with a sample containing the target of interest (incubation time: 60 min). After performing a series of washes to remove unbound materials, a biotinylated detection antibody specific for a different epitope on the target is added to the beads (incubation time: 60 min). The result is the formation of a sandwich of antibodies around the specific target. The reaction mixture is detected by the addition of a reporter dye, streptavidin-phycoerythrin (SA-PE), which binds to the sandwich complexes via the biotinylated detection antibodies (incubation time: 30 min). The contents of each well are drawn up into the Bio-Plex[®] array system, which identifies and quantifies each specific reaction based on bead color and fluorescence signal intensity (Figure 1). The magnetic beads are employed to enable automation of wash steps using a Bio-Plex Pro wash station and to ensure compatibility with all Luminex-based life science research instruments. Data acquisition was performed using the Bio-Plex[®] 200 system at low PMT setting, the Bio-Plex 3D system at standard PMT setting, or the Bio-Plex[®] MAGPIX[™] system using the default setting.

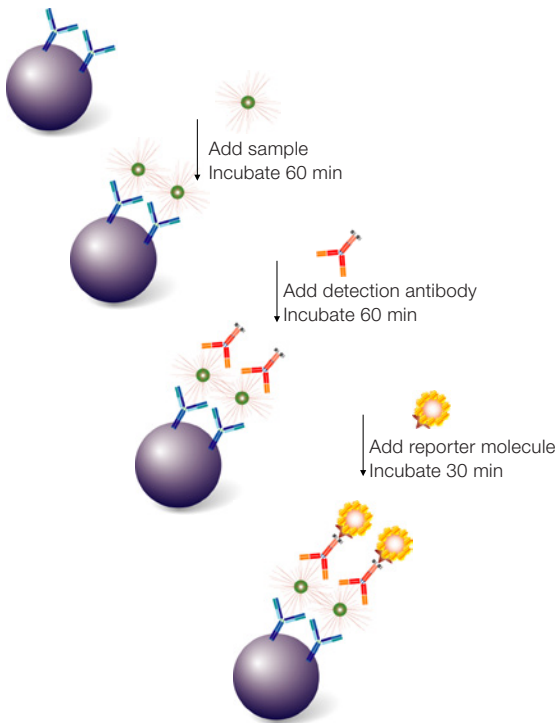


Fig. 1. Schematic representation of a Bio-Plex Pro RBM kidney toxicity assay workflow.

The performance of these assays was evaluated according to standard verification and validation parameters, including assay sensitivity, precision, accuracy, working ranges, specificity, and linearity of dilutions. The assays were further evaluated on urine samples collected from human, canine, or rat with various kidney-related disorders.

Assay Performance and Quality

Assay specificity was examined by performing single-detection cross-reactivity using selected standard points and native (spiked urine) matrices. The study was conducted by testing the individual detection antibody in the presence of multiplexed antigens and capture beads. The degree of cross-reactivity is defined as the percentage of signal detected relative to the specific signal for that analyte (Table 2). The findings were in agreement with studies carried out in spiked urine samples.

Table 2. Single-detection cross-reactivity based on standard point 1.

Detection	Capture Antibody					
	Calbindin	Clusterin	GST-π	IL-18	KIM-1	MCP-1
Calbindin	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Clusterin	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
GST-π	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
IL-18	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
KIM-1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
MCP-1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Human Kidney Toxicity Panel 2

Detection	Capture Antibody					
	Albumin	B2M	Cystatin C	NGAL	Osteopontin	TFF3
Albumin	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%
B2M	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Cystatin C	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
NGAL	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Osteopontin	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
TFF3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Rat Kidney Toxicity Panel 1

Detection	Capture Antibody				
	Clusterin	IL-18	KIM-1	MCP-1	Osteopontin
Clusterin	0.0%	0.9%	0.0%	0.0%	0.0%
IL-18	0.0%	0.0%	0.0%	0.0%	0.0%
KIM-1	0.0%	0.0%	0.0%	0.0%	0.0%
MCP-1	1.2%	0.7%	0.0%	0.0%	0.0%
Osteopontin	3.4%	6.0%	0.0%	0.0%	0.0%

Rat Kidney Toxicity Panel 2

Detection	Capture Antibody			
	B2M	Calbindin	Cystatin C	NGAL
B2M	0.0%	0.0%	0.0%	0.3%
Calbindin	0.0%	0.0%	0.0%	0.0%
Cystatin C	0.5%	0.0%	0.0%	0.1%
NGAL	0.0%	0.0%	0.0%	0.0%

Canine Kidney Toxicity Panel 1

Detection	Capture Antibody			
	Clusterin	KIM-1	MCP-1	NGAL
Clusterin	0.1%	0.0%	0.0%	0.0%
KIM-1	0.0%	0.0%	0.0%	0.0%
MCP-1	0.0%	0.0%	0.0%	0.0%
NGAL	0.0%	0.0%	0.0%	0.0%

Assay sensitivity, defined as limit of detection (LOD), was determined by adding two standard deviations to the average of the median fluorescence intensity (MFI) for ten replicates of the standard curve blank run on three separate plates. The value was converted to concentration as interpolated from the standard curve (Table 3).

Table 3. Limit of detection (LOD) in ng/ml.

Human Kidney Toxicity Panel 1					
Calbindin	Clusterin	GST-π	IL-18	KIM-1	MCP-1
2.698	0.041	0.152	0.005	0.007	0.002

Human Kidney Toxicity Panel 2					
Albumin	B2M	Cystatin C	NGAL	Osteopontin	TFF3
0.830	0.016	0.075	0.026	1.531	0.012

Rat Kidney Toxicity Panel 1				
IL-18	MCP-1	KIM-1	Osteopontin	Clusterin
0.181	0.049	0.006	0.0005	0.189

Rat Kidney Toxicity Panel 2				Rat Kidney Toxicity Albumin	
B2M	Calbindin	Cystatin C	NGAL	Albumin	Albumin
0.830	0.016	0.075	0.026	0.012	

Canine Kidney Toxicity Panel 1				Canine Kidney Toxicity Albumin	
MCP-1	Clusterin	NGAL	KIM-1	Albumin	Albumin
0.001	0.258	0.010	0.004	0.001	

Precision was calculated by testing urine-based controls in triplicate over five independent runs (Table 4). These runs were performed over a minimum of three days, with at least three different operators. The average of all coefficients of variation (%CV) of the calculated concentrations of the five runs was reported for intra-assay precision. The %CVs of all the calculated concentrations of the five runs was used to derive inter-assay precision.

Table 4. Intra- and inter-assay concentration (ng/ml) %CV.

Human Kidney Toxicity Panel 1							
		Calbindin	Clusterin	GST-π	IL-18	KIM-1	MCP-1
Intra-assay %CV	C1	4.7	1.5	0.7	5.7	1.1	1.4
	C2	0.8	6.2	2.0	2.6	1.8	3.3
Inter-assay %CV	C1	4.6	8.3	11.9	3.0	12.9	1.7

Human Kidney Toxicity Panel 2							
		Albumin	B2M	Cystatin C	NGAL	Osteopontin	TFF3
Intra-assay %CV	C1	1.0	0.1	0.5	4.0	2.0	1.5
	C2	4.9	2.7	1.3	3.9	3.0	3.8
Inter-assay %CV	C1	1.0	1.5	5.2	6.2	3.1	6.4

Rat Kidney Toxicity Panel 1

		IL-18	MCP-1	KIM-1	Osteopontin	Clusterin
Intra-assay %CV	C1	1.2	5.8	4.4	4.6	2.4
	C2	9.6	3.5	1.0	6.5	2.8
Inter-assay %CV	C1	3.8	2.0	3.5	9.4	2.4

Rat Kidney Toxicity Panel 2

Rat Kidney Toxicity Panel 2					Rat Kidney Toxicity Albumin	
		B2M	Calbindin	Cystatin C	NGAL	Albumin
Intra-assay %CV	C1	3.2	8.1	2.9	1.7	5.2
	C2	1.8	3.5	19.9	7.5	4.0
Inter-assay %CV	C1	16.7	0.8	5.3	13.4	22.6

Canine Kidney Toxicity Panel 2

Canine Kidney Toxicity Panel 2					Canine Kidney Toxicity Albumin	
		MCP-1	Clusterin	NGAL	KIM-1	Albumin
Intra-assay %CV	C1	0.6	4.5	0.5	4.4	7.8
	C2	1.3	4.0	3.4	3.4	1.3
Inter-assay %CV	C1	7.7	6.6	2.0	6.5	9.9

Note: C1 and C2 refer to level one and two controls provided in the kits.

Assay accuracy (also defined as recovery) was calculated as the percentage of the observed concentration value of a spiked standard of known concentration relative to the expected value. Overall, these assays recovered within 80–120% on at least seven of the eight standard points (Table 5). The accuracy of the assays was also demonstrated by the performance of a two-level urine-based control (C1 and C2) shown in Table 6. The test value obtained by the end user fell within the concentration range of the low and high control concentrations. These quality controls can be used to monitor replicate analysis, show consistency in reagent dispensing technique, and determine storage condition of the assay reagents.

Table 5. Assay accuracy (standard recovery).

Human Kidney Toxicity Panel 1						
	Calbindin	Clusterin	GST-π	IL-18	KIM-1	MCP-1
S1	101%	101%	103%	101%	100%	98%
S2	98%	99%	97%	98%	101%	104%
S3	103%	102%	104%	102%	100%	103%
S4	98%	99%	98%	100%	98%	98%
S5	99%	99%	99%	100%	102%	98%
S6	103%	101%	109%	98%	102%	104%
S7	102%	100%	94%	102%	95%	97%
S8	–	100%	–	99%	106%	85%

continues

Human Kidney Toxicity Panel 2

	Albumin	B2M	Cystatin C	NGAL	Osteopontin	TFF3
S1	99%	101%	100%	101%	101%	100%
S2	105%	99%	99%	99%	99%	99%
S3	102%	100%	101%	101%	100%	102%
S4	95%	100%	99%	99%	101%	99%
S5	106%	99%	100%	100%	98%	98%
S6	105%	103%	102%	103%	103%	103%
S7	86%	97%	94%	84%	96%	104%
S8	—	96%	—	—	104%	90%

Rat Kidney Toxicity Panel 1

	IL-18	MCP-1	KIM-1	Osteopontin	Clusterin
S1	99%	100%	100%	98%	99%
S2	99%	100%	102%	104%	101%
S3	106%	101%	98%	99%	99%
S4	94%	100%	101%	99%	103%
S5	119%	101%	101%	103%	98%
S6	83%	98%	100%	98%	101%
S7	97%	100%	101%	100%	99%
S8	106%	102%	98%	101%	103%

Rat Kidney Toxicity Panel 2

	B2M	Calbindin	Cystatin C	NGAL	Albumin
S1	110%	100%	100%	103%	100%
S2	94%	100%	100%	98%	100%
S3	99%	99%	101%	102%	100%
S4	103%	102%	98%	100%	100%
S5	98%	97%	98%	99%	102%
S6	103%	104%	108%	101%	98%
S7	82%	98%	94%	101%	100%
S8	—	96%	92%	99%	101%

Canine Kidney Toxicity Panel 1

	B2M	Calbindin	Cystatin C	NGAL	Albumin
S1	100%	101%	100%	100%	99%
S2	103%	99%	101%	100%	103%
S3	97%	102%	101%	102%	98%
S4	97%	99%	99%	98%	103%
S5	111%	100%	98%	100%	99%
S6	113%	104%	105%	103%	101%
S7	113%	95%	98%	100%	120%
S8	—	103%	100%	95%	100%

Note: Cells with a dash mark indicate recovery outside 80–120%.

Table 6. Performance characteristics of kit controls. All values are listed in ng/ml.

Human Kidney Toxicity Panel 1

	Calbindin	Clusterin	GST-π	IL-18	KIM-1	MCP-1
C1 range	138.00 414.00	45.00 134.00	17.00 50.00	1.50 4.50	1.40 4.20	0.43 1.30
Test value	245.28	85.40	31.66	2.85	2.76	0.81
C2 range	14.00 42.00	2.50 7.60	1.30 4.00	0.06 0.18	0.06 0.16	0.02 0.05
Test value	26.44	5.26	2.73	0.11	0.11	0.03

Human Kidney Toxicity Panel 2

	Albumin	B2M	Cystatin C	NGAL	Osteopontin	TFF3
C1 range	33.00 98.00	1.20 3.60	2.80 8.40	1.80 5.50	136.00 408.00	4.70 14.00
Test value	63.85	2.29	5.92	3.57	263.84	9.13
C2 range	3.90 12.00	0.10 0.31	0.31 0.94	0.15 0.45	4.80 15.00	0.24 0.71
Test value	7.58	0.21	0.63	0.31	10.00	0.46

Rat Kidney Toxicity Panel 1

	IL-18	MCP-1	KIM-1	Osteopontin	Clusterin
C1 range	3.10 9.40	4.20 13.00	3.60 11.00	0.12 0.37	89.00 266.00
Test value	5.46	8.45	6.78	0.24	177.87
C2 range	0.280 0.85	0.290 0.87	0.170 0.51	0.007 0.021	6.000 18.00
Test value	0.60	0.66	0.39	0.020	13.35

Rat Kidney Toxicity Panel 2

	B2M	Calbindin	Cystatin C	NGAL	Albumin
C1 range	279.64 838.92	73.05 219.16	3.94 11.83	41.03 123.09	4.10 12.00
Test value	505.81	141.77	7.83	75.80	8.36
C2 range	15.61 46.84	2.63 7.90	0.18 0.53	1.21 3.62	0.079 0.24
Test value	31.40	5.30	0.29	2.22	0.20

Canine Kidney Toxicity Panel 1

	MCP-1	Clusterin	NGAL	KIM-1	Albumin
C1 range	0.33 1.00	97.00 292.00	1.60 4.90	0.89 2.70	0.68 1.20
Test value	0.69	169.70	3.15	1.67	0.80
C2 range	0.02 0.07	2.40 7.20	0.03 0.10	0.03 0.10	0.02 0.04
Test value	0.04	5.33	0.08	0.07	0.03

Note: C1 and C2 refer to level one and two controls provided in the kits.

Assay working range is defined as the range between the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ) in which an assay is both precise and accurate. LLOQ and ULOQ were defined as the point at which the CV for samples was $\leq 30\%$ and $\leq 20\%$ respectively, with an accuracy of 80–120%. The results are tabulated in Table 7.

Table 7. Assay working range (ng/ml).

Human Kidney Toxicity Panel 1						
	Calbindin	Clusterin	GST- π	IL-18	KIM-1	MCP-1
LLOQ	4.36	1.26	0.50	0.02	0.02	0.01
ULOQ	1,750	1,250	230	15	21	3.8

Human Kidney Toxicity Panel 2						
	Albumin	B2M	Cystatin C	NGAL	Osteopontin	TFF3
LLOQ	11.22	0.01	0.14	0.07	1.75	0.02
ULOQ	640	22	40	34	2,100	98

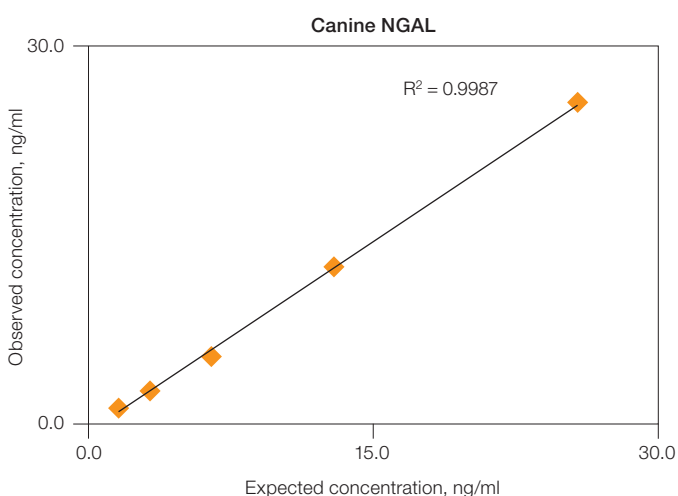
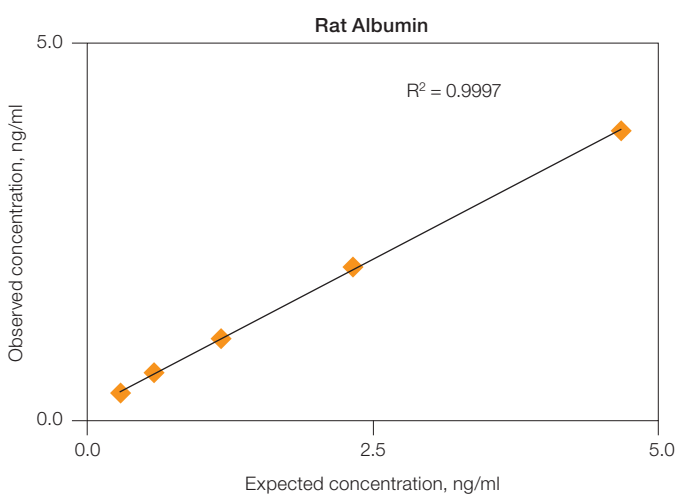
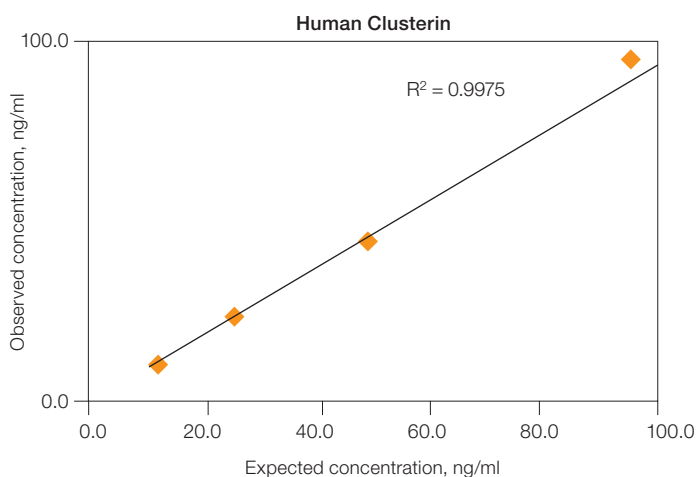
Rat Kidney Toxicity Panel 1					
	IL-18	MCP-1	KIM-1	Osteopontin	Clusterin
LLOQ	0.36	0.15	0.04	0.00	0.99
ULOQ	35	35	50	3.2	840

Rat Kidney Toxicity Panel 2				Rat Kidney Toxicity Albumin	
	B2M	Calbindin	Cystatin C	NGAL	Albumin
LLOQ	8.31	0.70	0.06	0.63	1.50
ULOQ	2,760	535	42	480	35

Canine Kidney Toxicity Panel 1				Canine Kidney Toxicity Albumin	
	MCP-1	Clusterin	NGAL	KIM-1	Albumin
LLOQ	0.01	0.69	0.01	0.01	0.01
ULOQ	4.8	800	9	10	4.8

Linearity of dilution ensures that analytes present in concentrations above the LLOQ can be diluted and measured accurately within the assay working range. In this study, a high control was serially diluted in standard diluent (Figure 2A) to obtain at least four data points. The observed and expected analyte concentrations were plotted to derive the correlation coefficient (R^2) values. The study was also conducted in urine samples collected from all species (Figure 2B).

A. Serially diluted control



B. Serially diluted urine

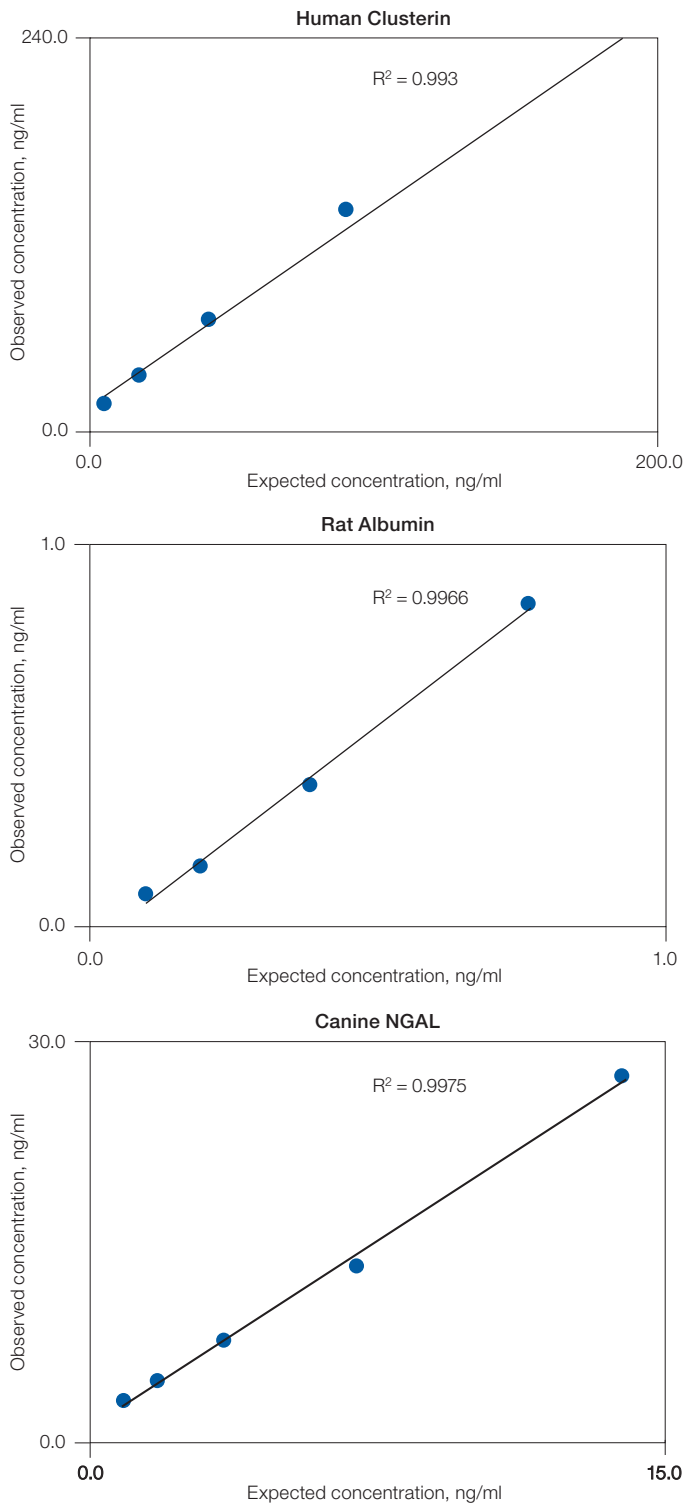


Fig. 2. Linearity of dilution. A high control (A) and urine samples (B) were serially diluted to obtain at least four data points. The observed and expected analyte concentrations were plotted and the correlation coefficient (R^2) values reflect linearity in signal response.

Parallelism was investigated by comparing the slope of a sample curve (spiked urine, serially diluted in urine matrix) with a standard curve prepared in standard curve diluent. The purpose is to ensure the sample matrix is biologically comparable to the matrix of the standard curve. This was judged by calculating the slope difference between the two curves (Figure 3).

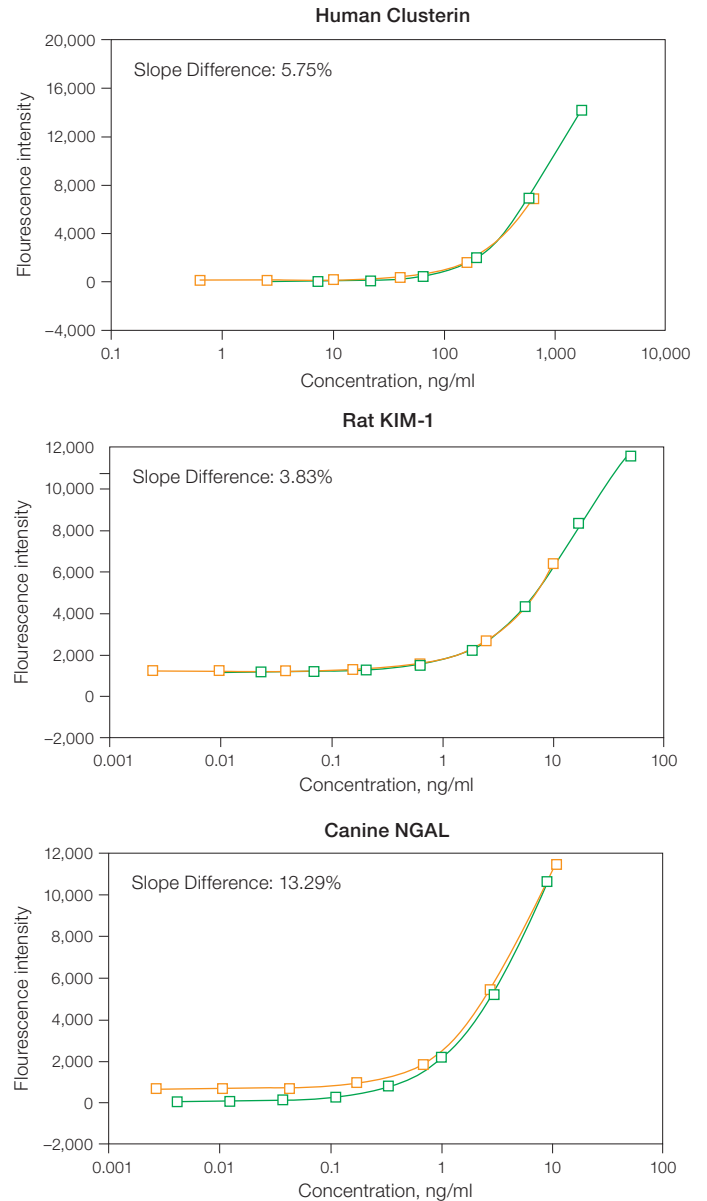


Fig. 3. Assay parallelism. Standard curve (—) and serial dilution (—) of a spiked urine sample.

Validation with Biological Samples

The kidney toxicity assays were validated primarily with urine samples. Sample dilution is unique for each of the assay panels (Table 8). Representative sample data are shown in Figure 4. The end users may also perform the same analysis in serum or plasma samples. In this case the dilution factor should be adjusted empirically for each of the targets or panels.

Table 8. Recommended sample dilution factor.

Sample Types	Human Kidney Toxicity		Rat Kidney Toxicity			Canine Kidney Toxicity	
	Panel 1	Panel 2	Panel 1	Panel 2	Albumin	Panel 1	Albumin
Urine	4	50	2	50	10000	15	10000
Serum/Plasma	User Defined						

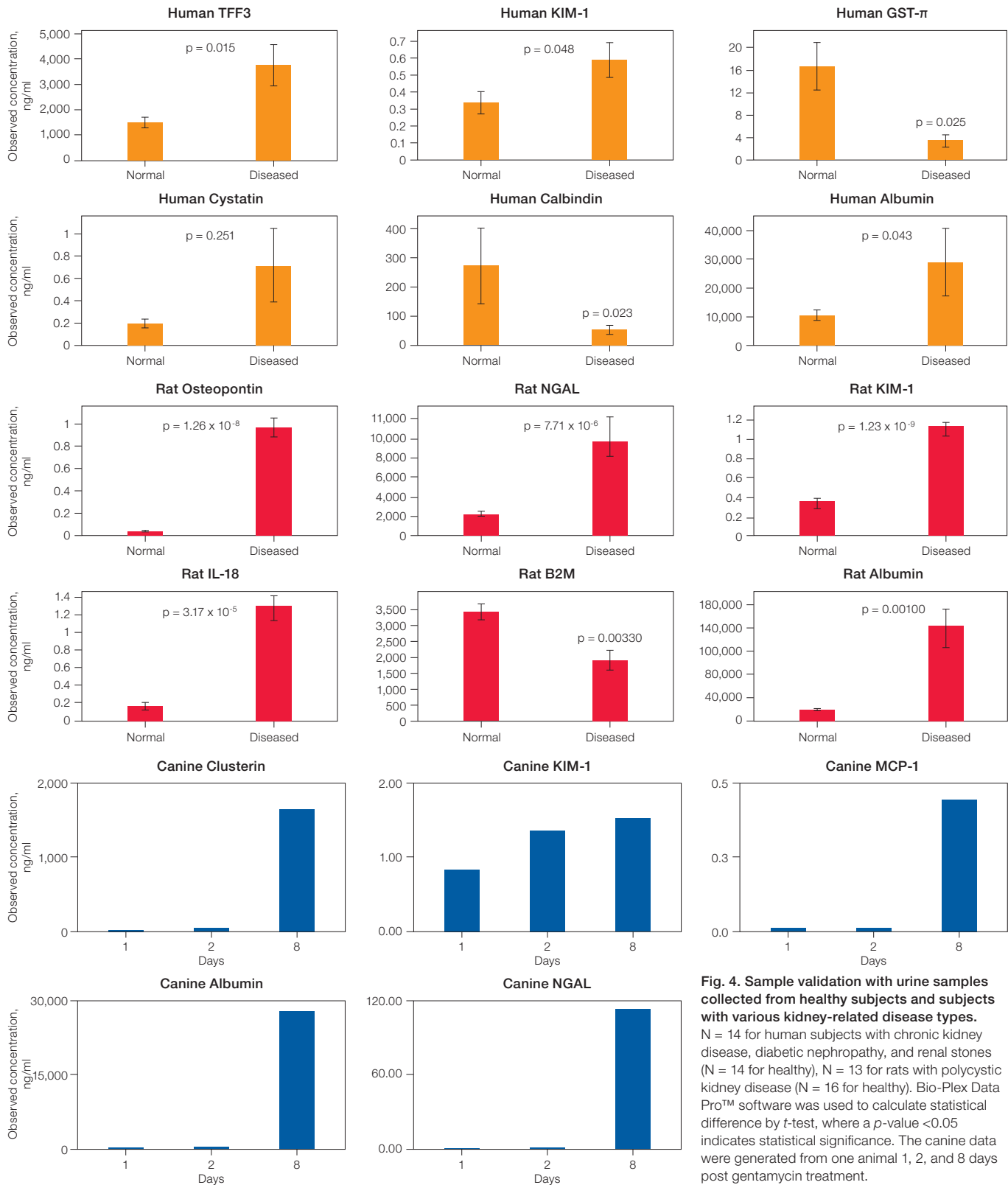


Fig. 4. Sample validation with urine samples collected from healthy subjects and subjects with various kidney-related disease types. N = 14 for human subjects with chronic kidney disease, diabetic nephropathy, and renal stones (N = 14 for healthy), N = 13 for rats with polycystic kidney disease (N = 16 for healthy). Bio-Plex Data Pro™ software was used to calculate statistical difference by *t*-test, where a *p*-value <0.05 indicates statistical significance. The canine data were generated from one animal 1, 2, and 8 days post gentamycin treatment.

Instrument alignment – Bio-Plex 200, Bio-Plex MAGPIX, and Bio-Plex 3D Systems

The assays were evaluated on Bio-Plex 200, Bio-Plex MAGPIX, and Bio-Plex 3D systems. Excellent agreement in sample readout was recorded on all three platforms (Figure 5).

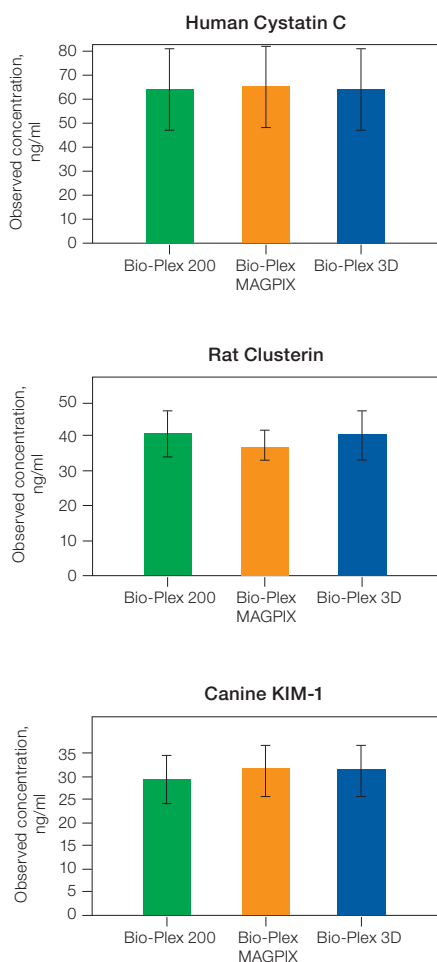


Fig. 5. Alignment in sample readout. Each bar represents analyte levels of normal urine samples collected from human (N = 15), rat (N = 16), and canine (N = 15), recorded by Bio-Plex 200 (■), Bio-Plex MAGPIX (■), and Bio-Plex 3D (■) systems.

Conclusions

The Bio-Plex Pro RBM kidney toxicity assays, developed in partnership with Myriad RBM, comprise a highly relevant set of biomarkers for early detection and characterization of kidney toxicity/injury. All seven assay panels demonstrated excellent sensitivity, broad working assay range, and high precision. We observed robust performance in human, canine, and rat urine samples across a range of physiological conditions — from normal to diseased to drug-treated. The multiplex format provides an effective solution for protein biomarker detection compared to traditional ELISA, and the premixed all-in-one kits enable drug development researchers to efficiently test lead compounds in both preclinical animal models and human clinical trials.

References

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- Slocum JL et al. (2012). Marking renal injury: can we move beyond serum creatinine? *Transl Res* 159, 277-289.

The Bio-Plex suspension array system includes fluorescently labeled microspheres and instrumentation licensed to Bio-Rad Laboratories, Inc. by the Luminex Corporation.

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