Sensitive, Early Detection of Kidney Toxicity in Preclinical Drug Safety

MILLIPLEX® MAP Rat Kidney Toxicity Multiplex Panels Measure Urinary Biomarkers of Kidney Injury

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Introduction

In conjunction with the liver, the kidney is an important site of drug metabolism, with many drugs and their soluble metabolites being eliminated in urine. Drug-induced toxicological findings are more common in the kidney than in most other organs. In order to sustain glomerular filtration and renal metabolism, the renal vascular bed receives a disproportionately large blood flow, averaging 20–25% of resting cardiac output.

One of the key considerations in drug development is acute kidney injury (AKI). Clinically referred to as acute renal failure, AKI is associated with a high mortality rate of up to 80%[1]. It is estimated that 19–33% of in-hospital AKI cases are attributed to drug-induced nephrotoxicity. Antibiotics, in particular aminoglycosides such as gentamicin and tobramycin, are the most frequently cited nephrotoxic drugs, followed by analgesics, NSAIDs, and contrast media. Other compounds that can potentially result in AKI include chemotherapeutic agents such as cisplatin and immunosuppressants such as cyclosporine[2]. The particular susceptibility of the kidney to drug toxicity can largely be attributed to its structure and function relating to its high rate of blood flow, concentrating and transport systems, and drug bioactivation mechanisms.
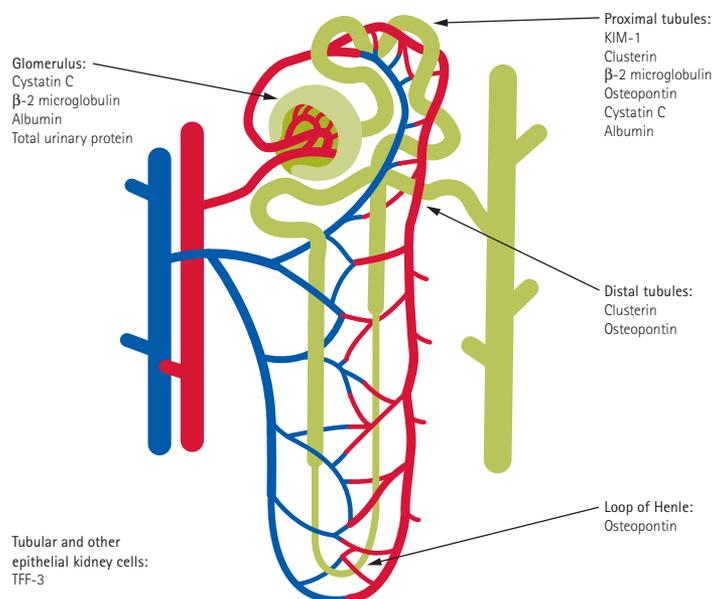
The most efficient way to avoid nephrotoxicity in clinical drug development is to have sensitive and specific biomarkers that can be studied in animal models, well before clinical studies are underway, enabling more timely diagnosis of AKI in humans and ideally localizing the injury to a specific nephron segment. Traditional biomarkers of renal function, including blood urea nitrogen (BUN) and serum creatinine (SCr), rely primarily on the detection of impaired kidney function. Both lack the sensitivity and specificity to adequately detect nephrotoxicity before significant loss of renal function[3]. SCr is a suboptimal marker following injury because levels often are not reflective of glomerular filtration rate due a number of renal and non-renal influences. The kidney has a large capacity to compensate for tubular injury, and global glomerular sclerosis, leading to nephron loss, can reach 50% before a functional parameter (such as change in creatinine clearance) is detected[4]. Similarly, BUN is not a reliable indicator of kidney injury because many other influences, including protein loading and volume status, affect BUN levels. Therefore, additional biomarkers that more sensitively predict toxicity in preclinical models and clinical situations are needed to enable drug developers to make more informed decisions during research and development.

Intensive efforts and resources have been devoted to the discovery and qualification of superior nephrotoxicity biomarker alternatives to SCr and BUN. Recently, several promising novel urinary biomarker candidates emerged, and

Figure 1.
Human nephron depicting primary structures of kidney toxicity. The depicted nephron segment-specific biomarkers of kidney injury are included as analytes in the MILLIPLEX MAP Kidney Toxicity Multiplex Panels.

Glomerulus:
Cystatin C
β-2 microglobulin
Albumin
Total urinary protein

Proximal tubules:
KIM-1
Clusterin
β-2 microglobulin
Osteopontin
Cystatin C
Albumin

Distal tubules:
Clusterin
Osteopontin

Loop of Henle:
Osteopontin

Tubular and other
epithelial kidney cells:
TFF-3

pooled data were submitted by the Predictive Safety Testing Consortium (PSTC) for regulatory decision[5-8]. The PSTC is a collaboration of scientists from the pharmaceutical industry, advisors from the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and academia to cross-qualify new safety biomarkers for drug development. The PSTC submission has resulted in the regulatory qualification of seven renal safety biomarkers for use in non-clinical and clinical drug development for detection of drug-induced renal toxicity[8]. The seven biomarkers endorsed by the PSTC are: kidney injury molecule-1 (KIM-1), albumin, total urinary protein, β-2-microglobulin, cystatin C, clusterin, and trefoil factor 3 (TFF-3). The performance of each biomarker was compared to SCr and BUN in time-course rat studies in the submission. With the exception of TFF-3, the new renal biomarkers outperformed the current standard biomarkers. These new biomarkers are able to detect earlier and more precise drug-induced kidney injury in preclinical studies and represent potential biomarkers for future clinical qualification. These qualification studies also found that measuring multiple biomarkers of kidney toxicity was more predictive of histopathological severity than individual biomarkers[9].

Several other biomarkers are still in exploratory phases but have been acknowledged as valuable candidate markers for AKI in several large studies. These studies have identified a relatively small number of genes that are specifically altered in acute renal tubule injury[10]. The front-runners under consideration are NAG (N-acetyl-β-D-glucosaminidase), osteopontin (OPN), glutathione-S-transferase, and neutrophil gelatinase-associated lipocalin (NGAL). These are low-molecular weight proteins, and increased levels of many of these proteins in urine indicate proximal tubule injury where a defect in proximal tubular uptake is reflective of cellular damage to the tubular epithelium. Continued and collaborative efforts toward biological qualification and analytical validation of these novel biomarkers by assay developers, drug developers, and regulatory agencies can decrease the late-stage failure rates of drug candidates due to kidney toxicity.

One challenge to measuring multiple biomarkers per animal in a research or preclinical setting is the limited amount of serum, urine or other sample. To meet this challenge, multiplexed immunoassays enable the investigator to conserve sample, measuring multiple biomarkers in a given volume instead of using large volumes of sample as is required in singleplex detection (ELISA, RIA, or immunoblotting). In this paper, we describe a method for multiplexed biomarker analysis of rat urine samples using the MILLIPLEX MAP Rat Kidney Toxicity Panels 1 & 2 on the Luminex® xMAP® platform, showing successful, simultaneous detection of PSTC-recommended biomarkers as well as the promising new biomarker, osteopontin (OPN).

Table 1.
Summary of claims submitted by the PSTC to the FDA and EMA on seven urinary safety biomarkers qualified as early indicators of kidney injury.

Biomarker	Qualified preclinically	Adds information to SCr and BUN	Outperforms SCr and/or BUN	Site of injury	Comments
KIM-1	Yes	Yes	Yes	Tubule	Levels may predict adverse outcome
Albumin	Yes	Yes	Yes	Glomerulus and tubule	Increased urinary excretion may reflect alterations in glomerular permeability and/or defects in proximal tubular reabsorption; limited specificity for AKI
Total urinary protein	Yes	Yes	Yes	Glomerulus	Glomerular damage functional marker
β-2-microglobulin	Yes	Yes	Yes	Glomerulus and tubule	Clinical applicability limited by instability in urine
Cystatin C	Yes	Yes	Yes	Glomerulus and tubule	Urinary levels may predict adverse outcome
Clusterin	Yes	Yes	Yes	Tubule	Increased urinary levels observed in rat models of tubular but not glomerular protein proteinuria
TFF-3	Yes	Yes	No	Various kidney compartments	Nephrotoxins cause decrease of TFF-3

Materials and Methods

Samples. Two different strains of rats were used in the presented rat toxicity studies. The gentamicin study uses the MILLIPLEX MAP Rat Kidney Toxicity Panel 2 (Cat. No. RKTX2-37K), with male Sprague Dawley® rats. Gentamicin was administered intraperitoneally at 0, 25, 50, or 75 mg/kg/day, to groups of five rats for 9 days. The salt study uses the MILLIPLEX MAP Rat Kidney Toxicity Panel 1 (Cat. No. RKTX1-37K), with Dahl salt-sensitive (Dahl/SS) rats. Dahl/SS rats were fed various percentage salt diets (0%, 4% or 8% NaCl) for 12 days.

Kidney Toxicity Biomarker Analysis: MILLIPLEX MAP Rat Kidney Toxicity Panels on the Luminex xMAP Platform. Safety biomarker analysis requires a platform that will reliably quantify these proteins from preclinical samples with sensitivity and specificity. ELISA assays have become the prevailing platform for accurate quantification, however, they have drawbacks with respect to throughput. In addition, preclinical study subjects in kidney toxicity are typically small rodents, therefore, there is a limitation upon the available sample volume.

To overcome these limitations, a number of emerging technology platforms, such as the Luminex xMAP platform, have been developed, making it possible to measure several proteins in a single sample robustly and rapidly[11]. Multiplexed analysis offers significant advantages with respect to time, reagent cost, sample requirements and the amount of data that can be generated. Combined with high quality antibody reagents and assay development expertise, multiplexed immunoassays on the Luminex xMAP platform can deliver the sensitivity, reproducibility, dynamic range (pg/mL to ng/mL), throughput and robustness demanded by biomarker analysis.

The MILLIPLEX MAP Rat Kidney Toxicity Panel 1 (Cat. No. RKTX1-37K) includes multiplexed assays for KIM-1, clusterin, and osteopontin (standard curves shown in Figure 2). This kit may used for the simultaneous quantification of all or any combination of the analytes in urine, and requires only 25 µL of neat urine sample volume.

The MILLIPLEX MAP Rat Kidney Toxicity Panel 2 (Cat. No. RKTX2-37K) includes multiplexed assays for albumin, β-2 microglobulin, and cystatin C (standard curves shown in Figure 3). This kit may be used for the simultaneous quantification of all or any combination of the analytes in dilute urine samples, and requires only 25 µL of diluted urine sample volume.

The results presented in this paper illustrate the application of MILLIPLEX MAP Rat Kidney Toxicity Panels 1 and 2 in a preclinical study.
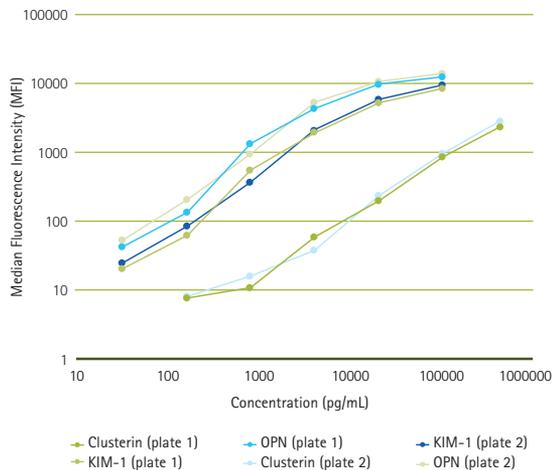

Legend: Clusterin (plate 1), KIM-1 (plate 1), OPN (plate 1), Clusterin (plate 2), KIM-1 (plate 2), OPN (plate 2)

Figure 2.

MILLIPLEX MAP Rat Kidney Toxicity Panel 1 (Cat. No. RKTX1–37K). Standard curves for clusterin, KIM-1, and osteopontin show that the assay is linear with sensitivity covering several orders of magnitude. The standards were incubated for 2 hours at room temperature on two different plates representing the dynamic range of the analytes.
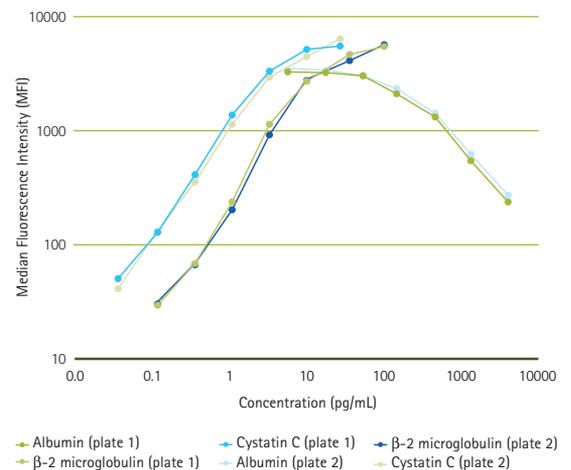

Legend: Albumin (plate 1), β-2 microglobulin (plate 1), Cystatin C (plate 1), Albumin (plate 2), β-2 microglobulin (plate 2), Cystatin C (plate 2)

Figure 3.

MILLIPLEX MAP Rat Kidney Toxicity Panel 2 (Cat. No. RKTX2–37K). Standard curves for albumin, β-2 microglobulin, and cystatin C show that the assay is linear and sensitive over several orders of magnitude. The standards were incubated for 2 hours at room temperature on two different plates representing the dynamic range of the analytes.

Data Normalization. Analyzing urinary biomarkers requires normalization of the concentration of the respective biomarker in urine for the degree of urine dilution. All data presented below were subjected to data normalization as described here. Analyte concentrations for all animals were first normalized by dividing by the corresponding urinary creatinine concentration. Urinary creatinine was measured with a clinical chemistry analyzer. All individual animal marker values normalized to creatinine were divided by the mean of the values in the concurrent control animals. Therefore, all marker values are shown as a fold-change versus the time-matched control group mean.

Results

Gentamicin nephrotoxicity in the Sprague Dawley rat.

Nephrotoxicity has been associated with a selective accumulation of gentamicin in the epithelial cells lining the proximal tubules after glomerular filtration. High doses of gentamicin in animals produces a typical pattern of nephrotoxicity, including increasing plasma concentrations of creatinine and urea, proteinuria, and substantially reduced creatinine clearance.

To assess the value of β-2-microglobulin for the detection of early stages of renal damage caused by gentamicin, rats were treated with various concentrations of gentamicin (0, 25, 50, or 75 mg/kg/d) for 7 days and analyzed with the MILLIPLEX MAP Rat Kidney Toxicity Panel 2 (Cat. No. RKTX2-37K) (Figure 4; data not shown for albumin and cystatin C).

Increased urinary excretion of filtered low-molecular weight proteins such as β-2-microglobulin is reflective of a primary defect in the proximal tubular uptake. Acute nephrotoxicity induced by a 7-day treatment with gentamicin resulted in a dose-dependent increase in urinary β-2-microglobulin (Figure 4). Increased secretion of β-2-microglobulin occurred with even the lowest dose of gentamicin (25 mg/kg/d). Significantly, such elevation of β-2-microglobulin correlated with severity of histopathologic changes observed in renal tubular degeneration and/or necrosis in the proximal tubes (data not shown). This supports previous findings that this biomarker provides added statistical value to the diagnosis of AKI from SCr and BUN levels alone[12].

Dahl Salt-Sensitive Blood Pressure Model.

The Dahl Salt-Sensitive (Dahl/SS) rat is used as a preclinical animal model of salt-induced hypertension and nephropathy. Rapid development of renal injury is a prominent feature

of salt-induced hypertension in Dahl/SS rats. Within a few weeks of high salt exposure, Dahl/SS rats develop substantial injuries in preglomerular vessels, arterioles, glomeruli, and the tubulointerstitial compartment. This prominence of renal injury in the Dahl/SS rat mimics human salt-sensitive forms of hypertension.

Dahl/SS rats were fed various percentage salt diets (0%, 4% or 8% NaCl) for 12 days to evaluate induced renal injury. Urinary samples collected daily after the onset of salt treatment were measured for clusterin, KIM-1 (data not shown), and osteopontin using the MILLIPLEX MAP Rat Kidney Toxicity Panel 1 (Cat. No. RKTX1-37K) (standard curves shown in Figure 2). Here, we show selected results for clusterin (Figure 5a) and osteopontin (Figure 5b). Subgroups of these rats were sacrificed at days 1, 2, 5 and 12 for histological examination.

Clusterin is induced in the kidney and urine after various forms of preclinical acute kidney injuries such as ischemia/reperfusion injury or toxicant-induced kidney injury. Clusterin, like KIM-1, is expressed in the dedifferentiated tubular cells after injury.
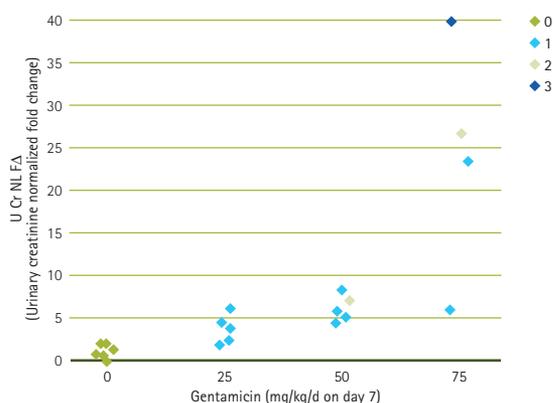

Figure 4.

Gentamicin administration results in increased urinary excretion of β-2-microglobulin. Rats were treated with various doses of gentamicin for 7 days. Urinary creatinine levels were measured on day 7. β-2-microglobulin levels are shown as fold-change relative to the average of concurrent control. The squares indicate individual animals. The severity grades of histopathologic change after gentamicin treatment are indicated on a scale of 0 (no observed pathology) to 3 with the indicated grades displayed as the following colors: grade 0 (green), grade 1 (bright blue), grade 2 (gray), and grade 3 (dark blue). The histomorphological change is shown at each necropsy day and vehicle-treated animals (control) are shown in green.

Urinary excretion of clusterin is increased with a high salt diet in Dahl/SS rats (Figure 5a), suggesting that salt-induced nephrotoxicity occurs in the tubules of the kidney. Mechanisms of salt-induced kidney injury are of interest; recent results include activation of mineralocorticoid receptor signaling as a possible mechanism[13].

Osteopontin is a secreted acidic protein with roles in the responses to a multitude of injurious stimuli. High OPN expression has been found in tissues with high cell turnover, and OPN up-regulation has been demonstrated in several models of renal injury, suggesting a possible role in tissue remodeling and repair. OPN is expressed by different epithelial cells of the kidney and induces monocyte/macrophage infiltration. Fold changes in urinary OPN relative to concurrent controls are clearly defined. As depicted in Figure 5b, a dramatic rise in urinary excretion of OPN was observed in this model of kidney injury induced by a high-salt diet.

In summary, the findings obtained with the Dahl/SS rat model demonstrated the utility of both clusterin and osteopontin as kidney injury biomarkers, indicating that, in this model, salt induces injury to kidney epithelial cells, particularly in the tubules. These findings have important potential clinical implications, since the effectiveness of antihypertensive therapies on the reduction of renal injury remains a point of appreciable need.
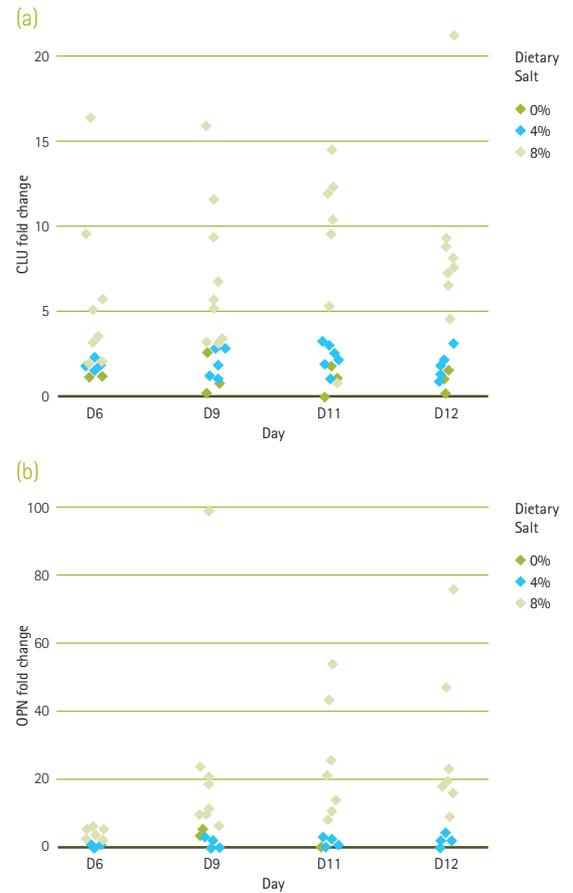

Figure 5.
Clusterin and osteopontin are markers of renal injury in the Dahl/SS Rat Model. Abundances of clusterin (a) or osteopontin (b) are shown as fold change relative to the average of concurrent controls. The squares represent individual animals. Animals were euthanized on day 5 for histological evaluation of toxicity and urine was collected from remaining animals on days 6, 9, 11 and 12.

Conclusion

Considerable progress has been made in identifying biomarkers of kidney injury. Urinary biomarkers hold substantial promise for monitoring potential adverse effects on kidney integrity and function[14,15]. Assays for measuring the new biomarkers and the acceptance of seven biomarkers for preclinical and translational drug development contexts by regulatory agencies offer a unique toolset to manage kidney safety in drug development settings and in routine clinical care. The extension of the biomarker qualification efforts into the evaluation of use of novel markers for renal injury in preclinical and clinical settings is of great importance for research and development across industry and academia.

In this study, various novel biomarkers of kidney injury were assessed in urine to determine their ability to detect the localization of acute kidney injury induced by gentamicin or high-salt diet. The presented data support the value of the novel rat kidney toxicity panels for improved discernment of kidney injury. The MILLIPLEX MAP Rat Kidney Toxicity Panels 1 & 2 are able to accommodate a broad context of study designs. The complexity of accumulating standardized preclinical data, interpreting the data, and ranking the new markers is a compelling reason to use biomarker panels which are comprised of several markers as opposed to inferring kidney injury based on a single biomarker. These panels enable researchers to measure multiple nephrotoxicity biomarkers in a very small sample volume, enhancing the predictive power of preclinical models while minimizing time, animal and reagent costs.

References

1. Trof, R.J., et al. Biomarkers of acute renal injury and renal failure. Shock. 2006; 26:245-253.

2. Parik, C.R., et al. New biomarkers of acute kidney injury. Critical Care Medicine. 2008; 36 (Suppl. 4): S159-165.

3. Pannu, N., et al. An overview of drug-induced acute injury. Critical Care Medicine 2008; 36 (Suppl. 4): S216-223.

4. Croker, B.P., et al. Dawson DV, Sanfilippo F. IgA nephropathy. Correlation of clinical and histologic features. Lab Invest. 1983 Jan;48(1):19-24.

5. Ferguson, M.A., et al. Biomarkers of nephrotoxic acute kidney injury. Toxicology. 2008; 245: 182-193.

6. Tesch, G.H., et al. Review: Serum and urine biomarkers of kidney disease: A pathophysiological perspective. Nephrology. 2010; 15: 609-616.

7. Bonventre, J.V., et al. Next generation biomarkers for detecting kidney toxicity. Nat. Biotechnol. 2010; 28: 436-440.

8. Editorial. Biomarkers on a roll. Nat. Biotechnol. 2010; 28: 431.

9. Yu, Y., et al. Urinary biomarkers trefoil factor 3 and albumin enable early detection of kidney tubular injury. Nat. Biotechnol. 2010; 28:470-477.

10. Kondo, C., et al. Identification of genomic biomarkers for concurrent diagnosis of drug-induced renal tubular injury using a large-scale toxicogenomics database. Toxicology. 2009; 265: 15-26.

11. Dieterle, F., et al. Monitoring kidney safety in drug development: Emerging technologies and their implications. Current Opinion in Drug Discovery & Development. 2008; 11: 60-71.

12. Dieterle, F., et al. Urinary clusterin, cystatin C, β-2-microglobulin and total protein as markers to detect drug-induced kidney injury. Nat Biotechnol. 2010 May;28(5):463-9.

13. Kawarazaki, H., et al. Mineralocorticoid receptor activation: a major contributor to salt-induced renal injury and hypertension in young rats. AJP Renal Physiol, in press. 2011.

14. Ozer, J.S., et al. A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. Nat. Biotechnol. 2010; 28: 486-494.

15. Mattes, W.B., et al. Translational Toxicology and the Work of the Predictive Safety Testing Consortium. Clinical Pharmacology & Therapeutics. 2009; 85: 327-330.

MILLIPLEX MAP Kidney Toxicity Assays

Reach faster, improved research decisions using multiplexed biomarker detection. Using Luminex xMAP technology, MILLIPLEX MAP multiplex panels are validated and optimized to yield maximum data from your precious rat or human samples.

Panel Name	Analytes	Sample Type	Catalogue Number
Rat Kidney Toxicity Panel 1	Clusterin, KIM-1, OPN	Urine (neat)	RKTX1–37K
Rat Kidney Toxicity Panel 2	Albumin, β-2-Microglobulin, Cystatin C	Urine (diluted)	RKTX2–37K
Human Kidney Toxicity Panel 1	KIM-1, OPN, Renin, TFF-3	Serum (neat)	HKTX1MAG-38K
Human Kidney Toxicity Panel 2	β-2-Microglobulin, Clusterin, Cystatin C	Serum (diluted)	HKTX2MAG-38K
Human Kidney Toxicity Panel 3	KIM-1, Renin, TFF-3	Urine (neat)	HKTX3MAG-38K
Human Kidney Toxicity Panel 4	Albumin, β-2-Microglobulin, Clusterin, Cystatin C, OPN	Urine (diluted)	HKTX4MAG-38K