Preclinical Evaluation of the Renal Toxicity of Oligonucleotide Therapeutics in Mice

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Abstract

Antisense oligonucleotides (ASO) therapeutics hold great promise for the treatment of numerous diseases, and several ASO drugs have now reached market approval, confirming the potential of this approach. However, some candidates have also failed, due to limited biodistribution/uptake and poor safety profile. In pursuit of better delivery and higher cellular uptake, ASO are being optimized, and new chemistries are developed or conjugated with various ligands. While these developments may lead to candidates with higher potency, it is important to keep the safety aspects in sight and screen for potential toxicity in early phases of preclinical development to avoid subsequent failure in clinical development. Our understanding of ASO-mediated toxicity keeps improving with increased preclinical and clinical data available. In this chapter, we will focus on the assessment of renal toxicity in mice and describe methods to measure the levels of general urinary biomarkers as well as acute kidney injury biomarkers following ASO treatment.

Key words Antisense oligonucleotides (ASO), Safety, Toxicology, Kidney toxicity, Acute kidney injury biomarker, Preclinical evaluation, Mouse model

1 Introduction

The field of synthetic antisense oligonucleotide (ASO) has advanced remarkably in the last decade, and ASOs represent a very promising therapeutic platform that keeps evolving rapidly, in particular in pursuit of delivery improvement. Many preclinical studies in the antisense area focus on improving ASO delivery and assessing their efficacy in target tissues, often neglecting the evaluation of toxicity, at least in early phases of development. However, safety assessment is particularly important when developing new generations of ASOs or novel delivery systems to avoid the potential failure of a new drug in further toxicological studies, as it happened with a peptide conjugated PMO (PPMO) targeting the human dystrophin exon 50 which was found to cause mild tubular degeneration in the kidneys of cynomolgus monkeys [1].

Toxicological properties of ASO have been comprehensively and extensively summarized previously [2, 3], and our understanding of them has allowed the development of predictive tests to select the best preclinical candidates.

Following systemic administration, the highest concentrations of ASO, independently of their chemistry, are found in liver and kidney, which are therefore considered as high-exposure organs. Importantly, tissue concentration does not further increase upon re-administration once steady-state is reached [2]. Accumulated ASOs can often be visualized at the histopathology level as basophilic granules on tissue sections stained with hematoxylin and eosin, but these effects are regarded as nonadverse because of their reversible nature upon treatment cessation. In rodents treated with high dose of PS-ASO, it is also frequent to observe tissue macrophages with a foamy appearance, referred to as histiocytes, which store cytokines in response to an activated state [3].

Considering the high concentrations of ASO accumulating in the kidneys, including the charge-neutral backbone such as PMO [4, 5], they are regarded as a common organ for toxicity. The highest uptake is generally observed in the proximal tubular epithelial cells of the convoluted tubule, whereas uptake in tubular cells in kidney medulla is much lower [6, 7]. Renal effects therefore tend to be more tubular than glomerular, apart from the reported glomerulopathies in mouse and monkey studies with the 2'OMe PS Dri-sapersen developed for the treatment of DMD [8]. However, it appears that this toxicity was linked to the chronic complement activation and inflammatory effects of the ASO and therefore over-predicted in animal studies since humans are less susceptible to these effects. Much more common are the lesions observed in the proximal tubules, which typically appear in animals treated with much higher doses of ASOs than the clinically relevant doses. Renal toxicity is mostly regarded as an accumulation-related toxicity and mostly sequence unspecific, except for more acute tubular lesions reported with high-affinity ASO such as locked nucleic acid (LNA) [9]. These effects might be related to excessive accumulation of RNase H-dependent off-target transcripts and/or specific protein binding [2] and a predictive EGF-based assay has recently been developed to exclude this type of kidney-toxic candidates [10].

Besides this EGF-based assay, several specific and early biomarkers of toxicity can be evaluated in mice (treated with high doses of ASO) to predict toxicity in preclinical development and exclude nephrotoxic candidates [8]. Evaluation of renal toxicology typically includes macroscopic examination of the kidneys upon necropsy of the animals followed by microscopic examination and careful histopathology analysis. General biomarkers of renal toxicity can be measured in the serum or plasma of treated mice such as urea, albumin, creatinine, and total protein. In this chapter, we focus on urinary biomarkers of kidney toxicity and describe the methods

to measure the levels of total protein, albumin, creatinine as well as specific kidney injury biomarkers as a way of evaluating the potential renal toxicity of antisense oligonucleotides in mice. For this assessment, urines are collected from ASO-treated mice either shortly after ASO injection to evaluate the potential acute kidney toxicity or after several weeks of repeated treatment to evaluate the potential long term renal toxicity induced by the accumulation of ASO in kidneys.

2 Materials

For all analysis described in this chapter, urine is collected using metabolic cages for rodents (e.g., metabolic cages for mice ref. 52–6731, Harvard apparatus, Holliston, MA).

2.1 Evaluation of Creatinine Levels in Urine

1. Creatinine assay kit (*see Note 1*).
2. Microplate reader capable of measuring absorbance at 490 nm.
3. Deionized or distilled water.
4. Test tubes for dilution of standards and samples.

2.2 Evaluation of Total Protein Level in Urine

1. Deionized or distilled water.
2. Acetone (pre-chilled overnight at -20°C).
3. Microplate reader capable of measuring absorbance at 562 nm.
4. Pierce BCA assay (*see Note 2*).
5. Test tubes for dilution of standards and samples.
6. Clear polystyrene microplates (96 well).

2.3 Evaluation of Albumin Level in Urine

1. Albumin ELISA kit (*see Note 3*).
2. Microplate reader capable of measuring absorbance at 450 nm.
3. Deionized or distilled water.
4. Test tubes for dilution of standards and samples.

2.4 Evaluation of Acute Kidney Injury Biomarkers (AKI) Level in Urine

1. Multiplex kidney injury panels (panel 1: MKI1MAG-94K and panel 2: MKI2MAG-94K, Merck-Millipore).
2. Luminex xMAP[®] technology compatible reader such as Luminex 200[™], HTS, FLEXMAP 3D[®] or MAGPIX[®] with xPONENT[®] software or Bio-Plex manager 6.1 software.
3. Automatic plate washer for magnetic beads (such as BioTek[®] 405 LS and 405 TS, EMD Millipore catalog #40–094, #40–095, #40–096, #40–097, or equivalent) or handheld magnetic separation block (EMD Millipore catalog #40–285 or equivalent).

4. Luminex sheath fluid (EMD Millipore catalog # SHEATH FLUID) or Luminex drive fluid (EMD Millipore catalog #MPXDF-4PK).
5. Adjustable pipettes with tips capable of delivering 25–1000 μL .
6. Multichannel pipettes capable of delivering 5–50 μL or 25–200 μL .
7. Reagent reservoirs.
8. Polypropylene microfuge tubes.
9. Rubber bands.
10. Aluminum foil.
11. Absorbent pads.
12. Laboratory vortex mixer.
13. Titer plate shaker (Lab-Line Instruments Model #4625 or equivalent).

3 Methods

3.1 Evaluation of Creatinine Levels in Urine

1. Collect urine using metabolic cages over 24 h, directly in refrigerated tubes (4 °C).
2. Record the total volume of collected urine.
3. Upon collection, centrifuge urines at $10,000 \times g$ for 10 min and aliquot supernatant.
4. Freeze aliquots at $-80\text{ }^{\circ}\text{C}$ for further analysis (Fig. 1) (*see Note 4*).

Urine creatinine is measured using a creatinine assay kit (in our case R&D Systems), based on the Jaffe reaction where creatinine is treated with an alkaline solution to yield a bright orange-red complex. Intensity of the color at 490 nm corresponds to the concentration of creatinine in samples.

1. Dilute the urine samples 20-fold as follows: 10 μL of urine sample + 190 μL of deionized or distilled water.
2. Prepare the alkaline picrate solution: for one plate, add 2.5 mL of NaOH to 12.5 mL of picric acid reagent (provided in the kit). Mix well. 100 μL of alkaline picrate solution is required per well.
3. Prepare the standard curve from the stock solution provided at 100 mg/dL. Label 7 microcentrifuge tubes from 20 mg/dL to 0.31 mg/dL. For the first point at 20 mg/dL, add 200 μL of stock into 800 μL of deionized or distilled water. The 20 mg/dL standard serves as the high standard. For the six subsequent tubes, serial dilute (1:2) by pipetting 500 μL of standard into 500 μL of deionized or distilled water made of 7 points from

Fig. 1 Schematic representation of urine collection possibilities following ASO treatment in mice and possible urine analysis

20 mg/dL to 0.31 mg/dL. Mix each tube thoroughly before the next transfer. Use deionized or distilled water as the zero standard (0 mg/dL).

4. Once all reagents, samples and creatinine standards are ready, remove excess microplate strips from the plate frame, return them to the foil pouch, and reseal.
5. Add 50 µL of standard, control, or sample to each well.
6. Add 100 µL alkaline picrate solution to each well.
7. Incubate for 30 ± 5 min at room temperature.
8. Determine the optical density of each well using a microplate reader set to 490 nm.
9. Calculate the creatinine concentration in your samples using the optical density measurements and the standard curve.
10. Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor (i.e., 20) to obtain the sample concentration.

3.2 Evaluation of Total Protein Level in Urine

1. Precipitate the protein in the urine samples by adding 40 µL dH₂O and 200 µL of prechilled acetone to 10 µL of urine.
2. Incubate samples at -20°C for 30 min, and then centrifuge 15 min at $14,000 \times g$ at 4°C .
3. Resuspend pellets in 40 µL of dH₂O.
4. Measure protein concentration using a BCA assay:

- (a) Prepare the standards from the 2 mg/mL albumin provided in the BCA assay, preferably using the same diluent as the one used for the samples. Nine standard tubes are prepared at concentrations 2000 (stock), 1500, 1000, 750, 500, 250, 125, 25, and 0 µg/mL of BSA.
- (b) Prepare the working reagent (WR). First, determine the total volume of WR required:

$$(n \text{ standards} + n \text{ unknowns}) \times (n \text{ replicates}) \times (\text{volume of WR per sample}) = \text{Total V required.}$$
 Prepare the WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1; A:B).
- (c) Then add 10 µL of each standard or unknown sample replicate into a microplate well (working range = 20–2000 µg/mL). Add 200 µL of WR to each well and mix plate thoroughly on a plate shaker for 30 s. Cover the plate and incubate at 37 °C for 30 min.
- (d) Cool the plate at room temperature.
- (e) Measure the absorbance at 562 nm on a plate reader.
- (f) Calculate the total protein concentration in your samples using the optical density measurements and the standard curve.

3.3 Evaluation of Albumin Levels in Urine

Albumin levels from urine samples are measured using an albumin ELISA kit. All reagents must be at room temperature before use. We describe the method below using the albumin ELISA kit from Bethy Laboratories:

1. Prepare 1× dilution buffer C by diluting 25 mL of 20× dilution buffer C into 475 mL of ultrapure water. Mix well.
2. Prepare standards: reconstitute the 900 ng albumin standard vial (included in the kit) with 1 mL of 1× dilution buffer C (all buffers mentioned are included in the kit) to achieve a final concentration of 900 ng/mL. Mix well. Label seven tubes, one for each standard curve point: 300, 100, 33.3, 11.1, 3.7, 1.23, and 0 ng/mL. Serially dilute 1:3 by adding 150 µL of the 900 ng/mL standard into the first tube containing 300 µL of 1× dilution buffer C. Mix well. Continue the dilution by adding 150 µL of the previous standard into 300 µL of 1× dilution buffer C in the next tube until the sixth tube. Last tube is the blank.
3. Prepare the urine samples. The recommended dilution is 1/1000. First perform a 1:25 dilution by adding 4 µL of urine to 96 µL of dilution buffer C, and then perform a 1:40 dilution by adding 6 µL of the 1:25 dilution into 234 µL of dilution buffer C.

4. Prepare the 1× wash buffer by diluting the 20× wash buffer in ultrapure water. For 1 L, dilute 50 mL of 20× wash buffer into 950 mL of ultrapure water.
5. Perform the assay: add 100 µL of standard or sample to designated wells (run each standard or sample in duplicate).
6. Cover the plate and incubate at RT for 1 h.
7. Gently remove the well content and wash the plate four times with 250 µL/well of 1× washing buffer.
8. Add 100 µL of anti-albumin detection antibody to each well and carefully attach a new adhesive plate cover. Incubate at RT for 1 h.
9. Gently remove the well content and wash the plate four times with 250 µL/well of 1× washing buffer.
10. Add 100 µL of HRP Solution A to each well and carefully attach a new adhesive plate cover. Incubate at RT for 30 min.
11. Gently remove well contents and wash the plate four times with 250 µL/well of 1× washing buffer. Blot off any residual liquid at the bottom of the wells.
12. Add 100 µL of TMB solution (included in the kit) to each well.
13. Do not cover the plate with a plate sealer.
14. Incubate the plate in the dark at RT for 30 min.
15. Stop the reaction by adding 100 µL of stop solution (included in the kit) to each well.
16. Measure absorbance on a plate reader at 450 nm.
17. Calculate the albumin concentration in your samples using the optical density measurements and the standard curve.

3.4 Evaluation of Acute Kidney Injury Biomarkers (AKI) Level in Urine

Acute kidney injury (AKI) biomarkers levels are analyzed by multiplex assays (MILLIPLEX® MAP) using the Luminex® technology.

The multiplex kidney injury panels (panel 1: MKI1MAG-94K and panel 2: MKI2MAG-94K, Merck-Millipore) are used according to the manufacturer's instructions to measure levels of β -2-microglobulin (B2M), renin, kidney injury molecule 1 (KIM-1), interferon-gamma induced protein 10 (IP-10), vascular endothelial growth factor (VEGF) (panel 1) and Cystatin C, epidermal growth factor (EGF), Lipocalin-2-NGAL, clusterin and osteopontin (OPN) (panel 2).

3.4.1 Evaluation of β 2-Microglobulin, Renin, Kim-1, IP-10, and VEGF Levels in Mouse Urines

β 2-Microglobulin, Renin, Kim-1, IP-10, VEGF are measured using the panel 1 (ref MKI1MAG-94K from Merck-Millipore).

1. Allow all reagents to warm to room temperature (20–25 °C) before use in the assay (except antibodies and beads).

2. Prepare the dilution of urine samples. For panel 1: dilute sample 1:25 in the assay buffer provided in the kit by adding 4 μL of urine to 96 μL of assay buffer.
3. Prepare the antibody-immobilized beads. For individual vials of beads, vortex for 1 min. Add 150 μL from each antibody-bead vial to the mixing bottle and bring final volume to 3.0 mL with assay buffer. Vortex the mixed beads well (*see Note 5*). There are 5 biomarkers in panel 1: add 150 μL from each of the five bead vials to the Mixing Bottle. Then add 2.25 mL assay buffer.
4. Prepare the quality controls (*see Note 6*):
 - (a) Reconstitute quality control 1 and quality control 2 with 250 μL deionized water.
 - (b) Invert the vial several times to mix and vortex.
 - (c) Allow the vial to sit for 5–10 min.
5. Prepare the standards:
 - (a) Reconstitute the mouse kidney injury panel 1 standard with 250 μL deionized water.
 - (b) Invert the vial several times to mix. Vortex the vial for 10 s.
 - (c) Allow the vial to sit for 5–10 min.
 - (d) This will be used as standard 7 (*see Note 6*).
 - (e) Label 6 Eppendorf microfuge tubes standard 1 through standard 6.
 - (f) Add 150 μL of assay buffer to each of the six tubes.
 - (g) Prepare serial dilutions (1:4) by adding 50 μL of the reconstituted standard to the standard 6 tube, mix well and transfer 50 μL of standard 6 to the standard 5 tube, mix well and transfer 50 μL of standard 5 to the standard 4 tube, mix well and transfer 50 μL of standard 4 to the standard 3 tube, mix well and transfer 50 μL of standard 3 to the standard 2 tube, mix well and transfer 50 μL of standard 2 to the standard 1 tube and mix well.
 - (h) The 0 pg/mL standard (background) will be assay buffer.
6. Perform the immunoassay procedure making sure that all reagents are warmed at room temperature (20–25 °C).
 - (a) Add 200 μL of assay buffer into each well of the plate. Seal and mix on a plate shaker for 10 min at room temperature (20–25 °C).
 - (b) Decant assay buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.

- (c) Add 25 μ L of each standard or control into the appropriate wells. Assay buffer should be used for 0 pg/mL standard (background).
 - (d) Add 25 μ L of sample (diluted) into the appropriate wells.
 - (e) Add 25 μ L of assay buffer to all wells.
 - (f) Add beads: vortex mixing bottle and add 25 μ L of the mix to each well (*see Note 7*).
 - (g) Seal the plate with a plate sealer.
 - (h) Wrap the plate with foil and incubate with agitation on a plate shaker (~700 rpm) overnight (16–18 h) at 2–8 °C.
 - (i) Place the plate on magnetic holder (handheld magnet, EMD Millipore Catalog #40–285) and rest plate on magnet for 60 s to allow complete settling of magnetic beads.
 - (j) Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid.
 - (k) Wash plate with 200 μ L of wash buffer by removing plate from magnet, adding wash buffer, shaking for 30 s, reattaching to magnet, letting beads settle for 60 s, and removing well contents as previously described after each wash. Repeat wash steps three times.
 - (l) Add 25 μ L of detection antibodies into each well. Allow the detection antibodies to warm to room temperature prior to addition.
 - (m) Seal, cover with foil and incubate with agitation on a plate shaker (~900 rpm) for 1 h at room temperature (20–25 °C). Do not aspirate after incubation.
 - (n) Add 25 μ L streptavidin-phycoerythrin to each well containing the 25 μ L of detection antibodies.
 - (o) Seal, cover with foil and incubate with agitation on a plate shaker for 30 min at room temperature (20–25 °C).
 - (p) Gently remove well contents (after placing the plate on magnetic holder) and wash plate three times following instructions listed above (**Step k**).
 - (q) Add 150 μ L of Sheath Fluid (or Drive Fluid if using MAGPIX[®]) to all wells. Resuspend the beads on a plate shaker for 5 min.
 - (r) Run plate on Luminex 200[™], HTS, FLEXMAP 3D[®] or MAGPIX[®] with xPONENT[®] or Bio-Plex manager 6.1 software.
7. Analysis: Save and analyze the median fluorescent intensity (MFI) data using a 5-parameter logistic or spline curve-fitting

method for calculating analyte concentrations in samples. For diluted samples, final sample concentrations should be multiplied by the dilution factor (25 as per protocol instructions). If using another dilution factor, multiply by the appropriate dilution factor.

3.4.2 Evaluation of Cystatin C, Epidermal Growth Factor (EGF), Lipocalin-2-NGAL, Clusterin, and Osteopontin (OPN) Level in Urine

Cystatin C, epidermal growth factor (EGF), Lipocalin-2-NGAL, Clusterin, and Osteopontin (OPN) levels are measured using the panel 2 (ref MKI2MAG-94K from Merck-Millipore):

1. Allow all reagents to warm to room temperature (20–25 °C) before use in the assay (except antibodies and beads).
2. Prepare the dilution of urine samples. For panel 1: dilute sample 1:1000 in the assay buffer provided in the kit by adding first 4 µL of urine to 96 µL of assay buffer, and then 2 µL of this dilution into 78 µL of assay buffer.
3. Prepare the antibody-immobilized beads:
 - (a) For individual vials of beads, vortex for 1 min.
 - (b) Add 150 µL from each antibody-bead vial to the mixing bottle and bring final volume to 3.0 mL with assay buffer.
 - (c) Vortex the mixed beads well (*see Note 5*).
 - (d) There are five biomarkers in panel 1: using five antibody-immobilized beads, add 150 µL from each of the five bead vials to the Mixing Bottle. Then add 2.25 mL assay buffer.
4. Prepare the quality controls:
 - (a) Reconstitute quality control 1 and quality control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex.
 - (b) Allow the vial to sit for 5–10 min (*see Note 6*).
5. Prepare the standards:
 - (a) First, reconstitute the mouse kidney injury panel 2 standard with 250 µL deionized water. Invert the vial several times to mix. Vortex the vial for 10 s. Allow the vial to sit for 5–10 min. This will be used as Standard 6 (*see Note 6*).
 - (b) Label 5 Eppendorf microfuge tubes standard 1 through standard 5.
 - (c) Add 150 µL of assay buffer to each of the five tubes.

- (d) Prepare serial dilutions (1:4) by adding 50 μL of the reconstituted standard to the standard 5 tube, mix well and transfer 50 μL of standard 5 to the standard 4 tube, mix well and transfer 50 μL of standard 5 to the standard 3 tube, mix well and transfer 50 μL of standard 3 to the Standard 2 tube, mix well and transfer 50 μL of standard 2 to the standard 1 tube and mix well.
 - (e) The 0 pg/mL standard (background) will be assay buffer.
6. Perform the immunoassay procedure making sure that all reagents are warmed at room temperature (20–25 °C).
- (a) Add 200 μL of assay buffer into each well of the plate. Seal and mix on a plate shaker for 10 min at room temperature (20–25 °C).
 - (b) Decant assay buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
 - (c) Add 25 μL of each standard or control into the appropriate wells. Assay buffer should be used for 0 pg/mL standard (background).
 - (d) Add 25 μL of sample (diluted) into the appropriate wells.
 - (e) Add 25 μL of assay buffer to all wells.
 - (f) Add beads: Vortex mixing bottle and add 25 μL of the mix to each well. During addition of beads, shake bead bottle intermittently to avoid settling.
 - (g) Seal the plate with a plate sealer.
 - (h) Wrap the plate with foil and incubate with agitation on a plate shaker (~700 rpm) overnight (16–18 h) at 2–8 °C.
 - (i) Place the plate on magnetic holder (handheld magnet, EMD Millipore Catalog #40–285) and rest the plate on magnet for 60 s to allow complete settling of magnetic beads.
 - (j) Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid.
 - (k) Wash plate with 200 μL of wash buffer by removing plate from magnet, adding wash buffer, shaking for 30 s, reattaching to magnet, letting beads settle for 60 s, and removing well contents as previously described after each wash. Repeat wash steps three times.
 - (l) Allow the detection antibodies to warm to room temperature.
 - (m) Add 25 μL of detection antibodies into each well.

- (n) Seal, cover with foil, and incubate with agitation on a plate shaker (~900 rpm) for 1 h at room temperature (20–25 °C). Do not aspirate after incubation.
 - (o) Add 25 µL streptavidin-phycoerythrin to each well containing the 25 µL of detection antibodies.
 - (p) Seal, cover with foil and incubate with agitation on a plate shaker for 30 min at room temperature (20–25 °C).
 - (q) Gently remove well contents (after placing the plate on magnetic holder) and wash plate three times following instructions listed above (**Step k**).
 - (r) Add 150 µL of sheath fluid (or drive fluid if using MAG-PIX[®]) to all wells. Resuspend the beads on a plate shaker for 5 min.
 - (s) Run plate on Luminex 200™, HTS, FLEXMAP 3D[®], or MAGPIX[®] with xPONENT[®] or Bio-Plex manager 6.1 software.
7. Analysis: Save and analyze the median fluorescent intensity (MFI) data using a five-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. For diluted samples, final sample concentrations should be multiplied by the dilution factor (1000 as per protocol instructions). If using another dilution factor, multiply by the appropriate dilution factor (*see* **Note 8**).

4 Notes

1. We describe methods using the creatinine assay kit from R&D Systems, including all reagents mentioned.
2. We describe methods using the Pierce BCA assay from Thermo Scientific, including all the reagents mentioned.
3. We describe methods using the albumin ELISA kit from Bethy Laboratories, including all the reagents mentioned.
4. Avoid multiple (>2) freeze/thaw cycles of urine samples. Urine samples should be aliquoted upon collection and just after centrifugation according to further analysis requirements (*see* Fig. 1). When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing, and centrifuge prior to use in the assay to remove particles.
5. The unused portion of prepared antibody-immobilized beads may be stored at 2–8 °C for up to 1 month.
6. The unused portion of prepared quality controls and standards may be stored at ≤ −20 °C for up to 1 month.

7. During addition of beads, shake bead bottle intermittently to avoid settling.
8. All the quantification methods described here measure the concentration of analyte (Albumin, KIM-1, etc.) in the collected urines (e.g., as ng/ μ L). Data can also be normalized to the levels of creatinine. Alternatively, data can be expressed as total quantity/24 h when the volume of urine collected in the metabolic cage (per 24 h) is taken into account.

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