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Development of a novel immunoassay for the simple and fast quantitation of neutrophil gelatinase-associated lipocalin using europium(III) chelate microparticles and magnetic beads

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ABSTRACT

Neutrophil gelatinase-associated lipocalin (NGAL) is a promising biomarker for diagnosing acute kidney injury (AKI). Currently, there are few assays for determining NGAL and they are complex, time-consuming or expensive. We aimed to establish an efficient immunoassay to measure NGAL in human urine simply and rapidly. A novel immunoassay for NGAL determination was established by combining a dissociation-enhanced-free time-resolved fluoroimmunoassay (TRFIA) and immunomagnetic separation. Based on a "sandwich"-type immunoassay format, analytes in samples were captured by a pair of monoclonal antibodies (mAb) in which one mAb was coated in magnetic beads and the other mAb was labeled with europium(III) chelate microparticles (CM-EUs) as "fluorescent reporters". NGAL concentrations were determined in a linear range (10–1500 ng mL^{-1}) with a limit of detection of 0.32 ng mL^{-1}. The reproducibility, recovery, and specificity of our TRFIA were acceptable. Our method was compared with that of a chemiluminescence immunoassay (CMIA) using 115 urine samples, and the results showed good correlation (R^2 = 0.8677). We expect our novel method to be useful for the early diagnosis of AKI.

1. Introduction

Neutrophil gelatinase-associated lipocalin (NGAL; lipocalin-2, siderocalin) is a small protein expressed in neutrophils and certain epithelia, including renal tubules (Mori and Nakao, 2007). Renal expression of NGAL is increased dramatically upon kidney injury, and NGAL is released into urine and plasma (Bataille et al., 2017). NGAL levels rise within 2 h of the insult, making NGAL an early and sensitive biomarker of kidney injury (Yukird et al., 2017).

Acute kidney injury (AKI) is attributed to an abrupt loss of kidney function that develops within 7 days (Mu et al., 2015). Clinical research has revealed that NGAL levels can be used to identify patients with AKI before diagnostic change in serum levels of creatinine (Devarajan, 2010a; Piccoli et al., 2012). Clinical evidence points to several benefits of NGAL testing: early diagnosis of AKI to admit earlier initiation of appropriate management; risk stratification of AKI; prediction of clinical outcomes (dialysis, duration of hospital stay, in-hospital death, mortality); lower hospitalization costs; monitoring the response to therapy (Devarajan, 2010a; Devarajan, 2010b; Clerico et al., 2012; Piccoli et al., 2012). Hence, determination of NGAL levels is of growing importance.

Several immunoassays for determining NGAL levels have been undertaken: research-grade enzyme-linked immunosorbent assay (ELISA) (BioPorto (Gentofte, Denmark); R&D Systems (Minneapolis, MN, USA)); chemiluminescent microparticle immunoassay (CMIA; Abbott Diagnostics, Abbott Park, IL, USA); immunoturbidimetry (Fellahi et al., 2017). ELISAs are accurate, but they are not suitable for clinical application because the analytical process requires ~4 h (Krzeminska et al., 2016). And a key limitation to CMIA applications is the availability and cost of the apparatus (Krzeminska et al., 2016). In addition, the accuracy of using immunoturbidimetry can be influenced by light intensity, as well as the distance between the detector and reaction cup (Lei et al., 2017). Thus, more fast and user-friendly methods are desired for the timely and popular measurement of NGAL levels.

Time-resolved fluoroimmunoassay (TRFIA) has been used widely in human diagnostics since it was first reported by Pettersson and Eskola in the 1980s (Lundin et al., 1994; Hou et al., 2012). By using lanthanide chelates as labels, this methodology possesses various advantages, such

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as long fluorescence lifetimes, low background signal, large Stoke's shift, narrow emission spectra, and no radioisotope pollution (Seemann et al., 1999; Andreadis et al., 2005; Hou et al., 2012).

However, the TRFIA mode used traditionally involves the coating of 96-well plates and labeling of europium (Eu) chelates, and has deficiencies. For example, the specific antigen or antibody is coated on the solid-phase plane, which does not enable easy and full contact with the analyte, thereby resulting in a longer reaction time. Moreover, the TRFIA used most widely is the dissociation-enhanced, lanthanide-induced fluorescence immunoassay (Hemmila et al., 1984), in which a nonfluorescent Eu(III) chelate is used as a label and the amount of Eu (III) measured through a step involving dissociation and fluorescence enhancement. The signal-development step of lanthanide-based immunoassays involves extra reaction procedures that are time-consuming and vulnerable to lanthanide contamination (Xu and Li, 2007).

Here, we proposed an immunoassay for NGAL detection using carboxylate-modified polystyrene Eu(III) chelate microparticles (CM-EUs) as labels and magnetic beads as coatings. The CM-EUs used in the present study replaced the conventional Eu chelate as the label. As special functional microspheres, CM-EUs incorporate thousands of fluorescent chelates within a single polystyrene shell to offer a high lanthanide-specific fluorescence together with enhanced labeling efficiency (Liang et al., 2015). In addition, the single polystyrene shell offers a stable environment to protect the chelates from interfering compounds, which coexist in the assay media (Kokko et al., 2007). More importantly, because of their intrinsic fluorescence, enhanced photostability and remarkable potential for signal amplification, the signal-development step becomes unnecessary and fluorescence can be measured immediately after immune reactions (Kokko et al., 2007; Xu and Li, 2007), which simplifies the experimental procedure.

In addition, NGAL monoclonal antibodies (mAb) were coupled to the surface of magnetic beads rather than immobilized on the surface of 96-well microplates. The magnetic beads suspended in the analytical solution provide a relatively large surface area (Hou et al., 2012). mAb-coated magnetic beads suspended in the reaction system form a homogeneous and stable liquid phase, which improves the efficiency of the reaction greatly. More antigens become accessible within a short time (Khare et al., 2004; Hou et al., 2012). Therefore, the binding reaction of antigen antibody can be more rapid, which reduces the analytical time further.

Thus, the purpose of our study was to use this novel method to achieve simple, fast, and accurate detection of NGAL. We evaluated the performance of our novel assay, including linearity, analytical sensitivity, reproducibility, recovery and specificity. A total of 115 urine samples were measured by the TRFIA we developed. These data were compared with those from a CMIA kit to demonstrate that our method was applicable for measuring NGAL in human urine.

2. Experimental

2.1. Reagents and apparatus

N-hydroxysulfosuccinimide (NHS), Tween-20, bovine serum albumin (BSA), Proclin-300 and 4-morpholineethanesulfonicacid (MES) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) and CM-EUs (ø = 200 nm) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Magnetic beads (code: MS300/Carboxyl) were from JSR Life Sciences (Tokyo, Japan). Anti-NGAL mAb (clone codes 5# and 7#) were obtained from Feipeng Biotechnology (Shenzhen, Guangdong, China) and NGAL antigen from Wantai Biopharm (Xiamen, China). A 1420 Multilabel Counter (Victor3™) were purchased from PerkinElmer Wallac (Turku, Finland). All other chemicals were of analytical reagent grade. Ultra-pure water was used throughout the study, and was produced using a Milli-Q™ water-purification system (Millipore, Bedford, MA, USA).

2.2. Solutions

Tris-buffered saline Tween-20 (TBST) solution (25 mmol L^{-1} Tris-HCl, 0.15 mol L^{-1} NaCl and 0.05% Tween-20 (*v/v*)) were used as blocking and preservation buffers (pH 7.2), respectively, for magnetic beads coated with anti-NGAL mAb. Activating buffer (25 mmol L^{-1} MES, pH 6.1), binding buffer for labeling (25 mmol L^{-1} phosphate buffer, pH 7.0), blocking buffer (25 mmol L^{-1} phosphate buffer, 2% BSA (*w/v*), pH 7.4), washing buffer (25 mmol L^{-1} Tris-HCl, 0.9% NaCl (*w/v*), 0.2% Tween-20 (*v/v*) and 0.05% proclin-300 (*v/v*), pH 7.8), labeling antibody dilution buffer (25 mmol L^{-1} Tris-HCl, 1% BSA (*w/v*), 5% trehalose (*w/v*), 20% sucrose (wt/vol) (*w/v*), and 0.05% proclin-300 (*v/v*), pH 9.0) and sample buffer (50 mmol L^{-1} Tris-HCl, 0.1% NaN_3 (*w/v*), 0.9% NaCl (*w/v*), 0.01% Tween-20 (*v/v*), 1.5% BSA (*w/v*), pH 7.8) were employed. All solutions were prepared fresh before use.

2.3. Preparation of CM-EUs coupled with anti-NGAL mAb

Conjugates of CM-EUs and anti-NGAL mAb (clone code 7#) were prepared. In brief, 2 mg of CM-EUs were suspended in 1 mL of activating buffer containing NHS and EDC with final concentrations of 10 mmol L^{-1} and 1.25 mmol L^{-1}, respectively. After reaction with gentle shaking for 30 min at room temperature, the mixture was washed with 1 mL binding buffer and centrifuged at 15000 × *g* for 20 min at 8 °C. After that the supernatant was removed. Then, the activated CM-EUs were washed twice and resuspended in 1 mL of binding buffer using sonication. Subsequently, 0.01 mg of anti-NGAL mAb (which was purified and condensed in advance using a centrifugal filter unit with an Ultracel-50 membrane) was mixed with activated CM-EUs, and then the reaction mixture was shaking gently and constantly for 2 h at room temperature. Uncoupled mAb were removed within centrifugation at 10000 × *g* for 15 min at 4 °C. After washing twice, 1 mL of blocking buffer was added and shaken for another 1 h at room temperature to block unreacted active sites. The supernatant was discarded while the conjugate was washed. After that the supernatant was sonicated using a microprobe at amplitude of 5% for 3 min, and the washing buffer was removed by centrifugation at 10000 × *g* for 15 min at 4 °C for three times. Finally, the conjugates were resuspended in 0.2 mL of labeling antibody dilution buffer to adjust a CM-EUs concentration of 10 g L^{-1}, and CM-EUs were stored at 4 °C until use (Liang et al., 2015).

2.4. Preparation of magnetic beads coated with anti-NGAL mAb

Conjugates of magnetic beads and anti-NGAL mAb (clone code 5#) were prepared. First, 10 mg of carboxyl-modified magnetic beads at a concentration of 100 mg mL^{-1} were washed five times using binding buffer and the supernatant was removed using a magnetic separator. Then, 25 μL of fresh EDC and 40 μL of NHS (both 10 mg mL^{-1}) solutions were added initially to 10 mg of carboxyl-modified magnetic beads in 1 mL of binding buffer to activate carboxyl groups on the surface of the magnetic beads. The mixture were rotated for 30 min at room temperature and then washed thrice with 1 mL of binding buffer. The supernatant was removed from the magnetic field. Next, 250 μg of anti-NGAL mAb in 1 mL of binding buffer were added to the activated magnetic beads and mixed by gentle and constant rotation for 18 h at room temperature. After the reaction, The washing process was repeated thrice to remove excess antibody and followed by blocking with 3 mL of TBST buffer for another 3 h at room temperature. After the final washing, the magnetic beads-mAb conjugates were stored in TBST buffer at 4 °C until use.

2.5. Sample preparation

The study protocol was approved by the ethics committee of the General Hospital of Guangzhou Military Region. The methods for sample collection and experiments were carried out in accordance with

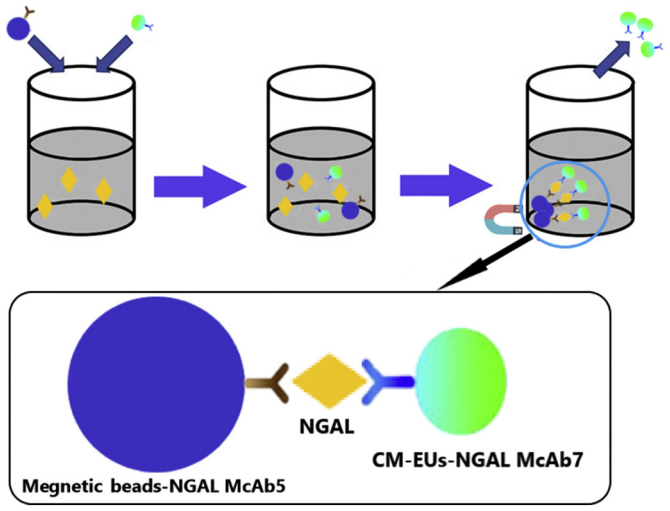

Fig. 1. Schematic illustration of the proposed immunoassay.

the principles stated in the *Declaration of Helsinki*. Written informed consent was obtained from each patient use of their samples.

A total of 115 urine samples were obtained from the General Hospital of Guangzhou Military Region (Guangzhou, China) and stored at −80 °C until use.

2.6. Assay procedure

The TRFIA was carried out as described in Fig. 1. First, 25 L of standards or urine samples (diluted 1:10 with sample buffer) were added to test tubes. Then, 25 µL of magnetic beads-NGAL mAb and 100 µL of CM-EUs-NGAL mAb were added stepwise. and the mixture was gently incubated for 1 h at 37 °C. Second, immune complexes were separated magnetically to remove the supernatant, subsequently being washed four times with the washing buffer. Finally, the resulting complexes were resuspended in 150 µL of washing buffer and time-resolved fluorescence intensity was measured immediately on a Victor3™ 1420 Multi-label Counter equipped with filters for Eu (613 nm).

3. Results and discussion

3.1. Dilution test

To confirm the reliability of the proposed method, three urine samples containing different levels of NGAL were diluted over the range 1 to 1/32. As shown in Fig. 2, three sample dilutions showed good linearity (R^2 = 0.999, 0.996, 0.999 for sample 1, sample 2, sample 3). Dilution test is used to evaluate whether the method is reliable. The samples were subjected to serial gradient dilution using suitable and selected diluents within the analytical range. When the dilution is linear, the fluorescence value of continuous dilution points will change linearly. After multiplying each dilution point by the dilution factor, the sample concentration will be approximately the same. For example, if the sample is doubly continuously diluted, the fluorescence values between each point should be approximately twice as different. If the sample does not exhibit linearity under serial dilution, it indicates that the matrix component interferes with the accurate detection of the target analyte at a given dilution. The result of dilution test demonstrated that the proposed method was feasible for quantitative measurements.

3.2. Analytical sensitivity and linear range

A standard curve for the TRFIA was carried out based on measurement with a series of NGAL standards (0, 10, 100, 500, 1000 and

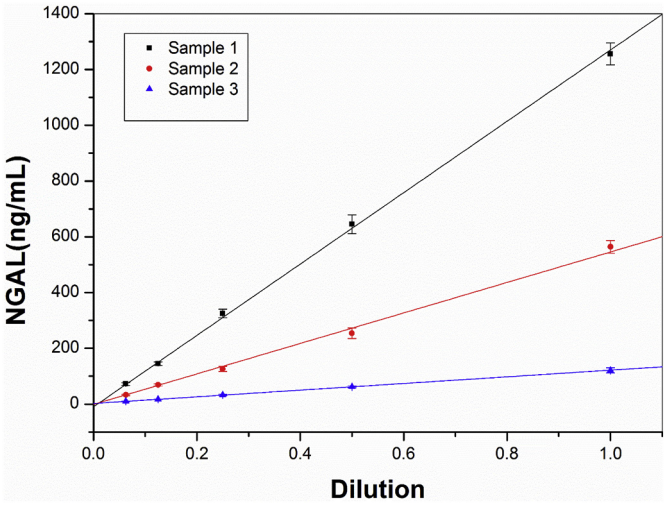

Fig. 2. Dilution linearity for NGAL based on measurement of three urine samples.

1500 ng mL^{-1}) formulated with NGAL antigen (1 mg mL^{-1}) and the concentration was detected by a CMIA kit (Abbott Diagnostics). The standard curve was represented by the equation:

$$\log(y) = 3.187 + 0.854\ \log(x)\ (R^2 = 0.99567)$$

Each value was based on five determinations. The correlation coefficient, 0.99567, signified excellent linearity for the range of concentrations investigated (Fig. 3). A Hook effect was not observed at high concentrations. The linear range of this assay was determined to be 10–1500 ng mL^{-1}. Compared with ELISA kits from BioPorto (10–1000 pg mL^{-1}) and R&D Systems (0.2–10 ng mL^{-1}), the proposed method exhibited a broad linear range within the additional advantages of shorter detection time (1 h; ELISAs need ≥4 h). And the detection time of the tradition TRFIA is > 2 h. This is due to the use of magnetic beads as the coatings. The magnetic beads are uniformly distributed in the reaction system. The surface area of magnetic beads is larger than that of the traditional 96-well microplates. It is easier for antigens and antibodies to come into contact, so it takes less time to reach a dynamic equilibrium. The limit of detection (defined as the concentration corresponding to zero standard fluorescence intensity plus two SD (n = 20)) was 0.32 ng mL^{-1}. The limit of detection of ELISA is 4 pg mL^{-1}. As a research-grade reagent, the limit of detection of the ELISA is much lower than our TRFIA. But it is not suitable for clinical

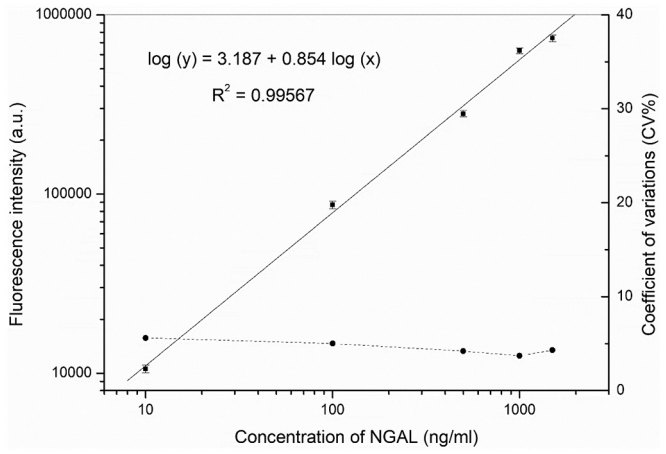

Fig. 3. Standard curve for the proposed TRFIA. The formula of the line of fit was log (y) = 3.187 + 0.854 log (x) with a R^2 of 0.99567 and intra-assay CV% for each data point based on five replicates.

Table 1
Intra-assay reproducibility and recovery of the TRFIA.

Theoretical value (ng mL^{-1})	Mean ± SD (ng mL mL^{-1})	CV (%) (n = 6)	Recovery (%)
20.000	20.618 ± 1.897	9.2	100.3%
200.000	209.861 ± 15.010	7.2	104.9%
1000.000	1096.095 ± 71.246	6.5	109.6%

detection of NGAL for the narrow linear range (10–1000 pg mL^{-1}). NGAL level in clinical samples are typically in the tens to hundreds of nanograms. A high degree of dilution is required for the sample detected by ELISA. It brings many difficulties and errors to the operation. Meanwhile, The limit of detection of CMIA is 0.7 ng mL^{-1}. The results suggested that the proposed method possessed high sensitivity and had potential clinical application for NGAL measurement.

3.3. Reproducibility and recovery of the TRFIA

The reproducibility of the TRFIA was evaluated by the intra- and inter-assay coefficient of variation (CV). Three concentrations of NGAL controls (20, 200 and 1000 ng mL^{-1}) were measured six times for intra-assay CV and three times independently for inter-assay CV. The intra-assay CV was 9.2%, 7.2% and 6.5%, respectively (Table 1). The inter-assay CV was 9.7%, 8.5% and 7.3%, respectively (Table 2). Hence, the intra-assay CV and inter-assay CV were < 10%. To investigate recoveries, three concentrations of controls were measured six times by the same method, respectively. The analytical recovery was calculated according to the following equation: Analytical recovery (%) = (Observed / Expected) × 100%. The general analytical recovery of the assay was good, with a recovery range from 100.3%–109.6% (Table 1). Thus, the reproducibility and recovery of the proposed TRFIA was acceptable for NGAL quantitation (Engling et al., 1995; Matsumoto et al., 1999).

3.4. TRFIA specificity

To evaluate the specificity of the TRFIA for NGAL, we measured the cross-reactivity with nine possible interfering compounds (matrix metalloproteinase (MMP)-2, MMP-3, MMP-7, MMP-9, MMP-14, β_2-microglobulin (β_2-MG), α-Microglobulin (α-MG), kidney injury molecule 1 (KIM-1), Cystatin C (Cys C)) which potentially coexist with NGAL if IKA are present (Mia Gebauer et al., 2012; DI Carlo, 2013; Ricci et al., 2015) (Table 3). There was no cross-reactivity with MMP-2, MMP-3, MMP-7, MMP-9, MMP-14, α-MG, KIM-1, Cys C or β_2-MG. These results indicated that the developed TRFIA is high specific to the NGAL antigen, and that it could be used for NGAL measurement in human urine.

3.5. Clinical application of the established assays

To further evaluate the feasibility of the proposed method for clinical applications, 115 urine samples were obtained. NGAL levels were measured by our proposed TRFIA and compared with the ARCHITECT Urine NGAL assay (Abbott Diagnostics) based on the CMIA. Linear regression analyses revealed good correlations between the proposed method and the CMIA (Fig. 4). The correlation coefficient was R^2 = 0.8677 between our TRFIA method and the commercial CMIA kit.

Table 2
Inter-assay reproducibility of the TRFIA.

Theoretical value (ng mL^{-1})	Mean ± SD (ng mL^{-1})	CV (%) (n = 18)
20.000	21.256 ± 2.062	9.7
200.000	205.452 ± 17.463	8.5
1000.000	1084.535 ± 79.171	7.3

Table 3
Specificity of the TRFIA for NGAL.

Compound	Concentration (ng mL^{-1})	Measured values (ng mL^{-1})	Cross-reactivity (%)
mmp-2	1000	0.49	0.049
mmp-3	1000	1.36	0.136
mmp-7	1000	0.58	0.058
mmp-9	1000	7.09	0.709
mmp-14	1000	1.16	0.116
β2-MG	1000	1.20	0.120
KIM-1	1000	1.25	0.125
α-MG	1000	0.75	0.075
Cys C	500	0.69	0.138

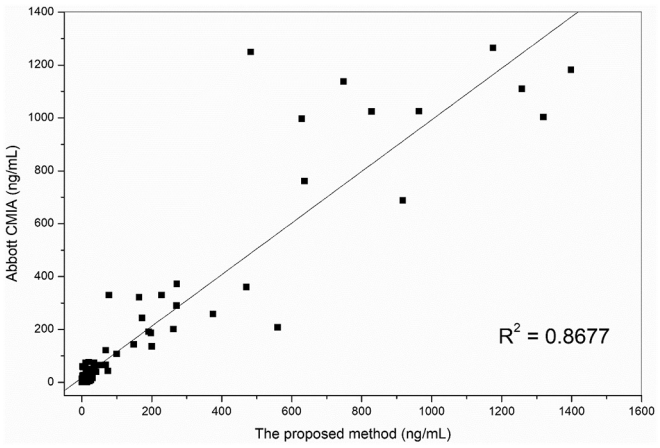

Fig. 4. Comparison of NGAL levels in 115 urine samples measured using our TRFIA and a CMIA kit. The correlation coefficient was R^2 = 0.8677.

The equation of the regression curve was y = 0.8895× + 2.0537 (where x is the NGAL concentration estimated with our TRFIA and y is the NGAL concentration from the CMIA).

Compared with CMIA from Abbott Diagnostics, our TRFIA showed a certain correlation, indicating that this method was suitable for clinical application. However, the results of some of the samples of our TRFIA deviated from that of the CMIA from Abbott Diagnostics (which is the most authoritative assay used currently in the clinic). The reasons for the reported differences may result from complex matrix of urine and different measurement technologies. More clinical samples must be tested to better analyze and further improve the accuracy and precision of our TRFIA. However, this method was simple and practical. Use of EU-CMs made the assay possess high sensitivity and low background values without the traditional time-resolved dissociation-enhancement step. And use of magnetic beads improved reaction speed. The organic combination of the two materials made the novel method possess superior performance. It is expected that this method will be a popular tool for monitoring NGAL levels in the clinic.

4. Conclusions

We used a double-antibody “sandwich” immunoassay based on magnetic beads and modified polystyrene CM-EUs to determine the NGAL concentration in human urine. The proposed method combines the advantages of using magnetic beads and lanthanide chelate particles. Therefore, a shorter detection time, simpler experimental operation, wide linear range and higher sensitivity were exhibited. The method established here showed good correlation with the CMIA kit from Abbott Diagnostics. We hope that this novel assay could be used to measure NGAL levels in human urine.

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