Clinica Chimica Acta 490 (2019) 55–62

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Measurement of urinary matrix metalloproteinase-7 for early diagnosis of acute kidney injury based on an ultrasensitive immunomagnetic microparticle-based time-resolved fluoroimmunoassay

Junyu Liang[a,1], Guanfeng Lin[b,1], Jianwei Tian[c], Jiejing Chen[d], Rongliang Liang[a], Zhenhua Chen[a], Qiaoting Deng[a], Zhining Dong[e], Tiancai Liu[a], Yingsong Wu[a,*]

[a] Institute of Antibody Engineering, School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangzhou, China
[b] Experimental Center of Teaching and Scientific Research, School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangzhou, China
[c] Division of Nephrology, Nanfang Hospital, Southern Medical University, National Clinical Research Center for Kidney Disease, State Key Laboratory of Organ Failure Research, Guangdong Provincial Institute of Nephrology, Guangzhou, Guangdong, China
[d] Nephrology Department of Guilin No.181 Hospital, Guangxi Key Laboratory of Metabolic Diseases Research, Guilin Key Laboratory of Kidney Diseases Research, Guilin, Guangxi, China
[e] Guangzhou Darui Biotechnology Co. LTD, Guangzhou, China

ARTICLE INFO

Keywords:
Acute kidney injury
Matrix metalloproteinase-7
Sensitive
Immunomagnetic microparticles
Time-resolved fluoroimmunoassay

ABSTRACT

The morbidity and mortality associated with acute kidney injury (AKI) remain obstinately high. Early diagnosis is urgently required and should be pursued in at-risk populations. Recently, a newly validated biomarker, matrix metalloproteinase-7 (MMP-7), was reported as a novel indicator for early AKI prediction and a noninvasive surrogate biomarker of kidney function. Monitoring urinary MMP-7 (uMMP-7) levels fills the gaps in early diagnosis of AKI at early onset. However, the lack of available reagents for its rapid detection limits its use. Herein, we established an ultrasensitive and rapid immunomagnetic microparticles-based time-resolved fluoroimmunoassay to measure urinary MMP-7 in AKI patients. The assay time is 30 min. The calibration curve showed high linear correlation (r = 0.9998) with a linearity of detection of 0.063–150 ng mL^{-1} and lower limit of detection of 0.039 ng mL^{-1}. The coefficient variation of the intra- and inter-assay lower than 5.17%, and the analytical recovery was 99.06%–105.60%. Testing of clinical samples using the proposed assay and a DUOSET@ ELISA kit showed good correlations in the comparison of uMMP-7 levels (r = 0.9541) and uMMP-7/uCreatinine (r = 0.9595). The proposed assay has satisfactory analytical performance and may serve as a promising tool for early diagnosis of AKI.

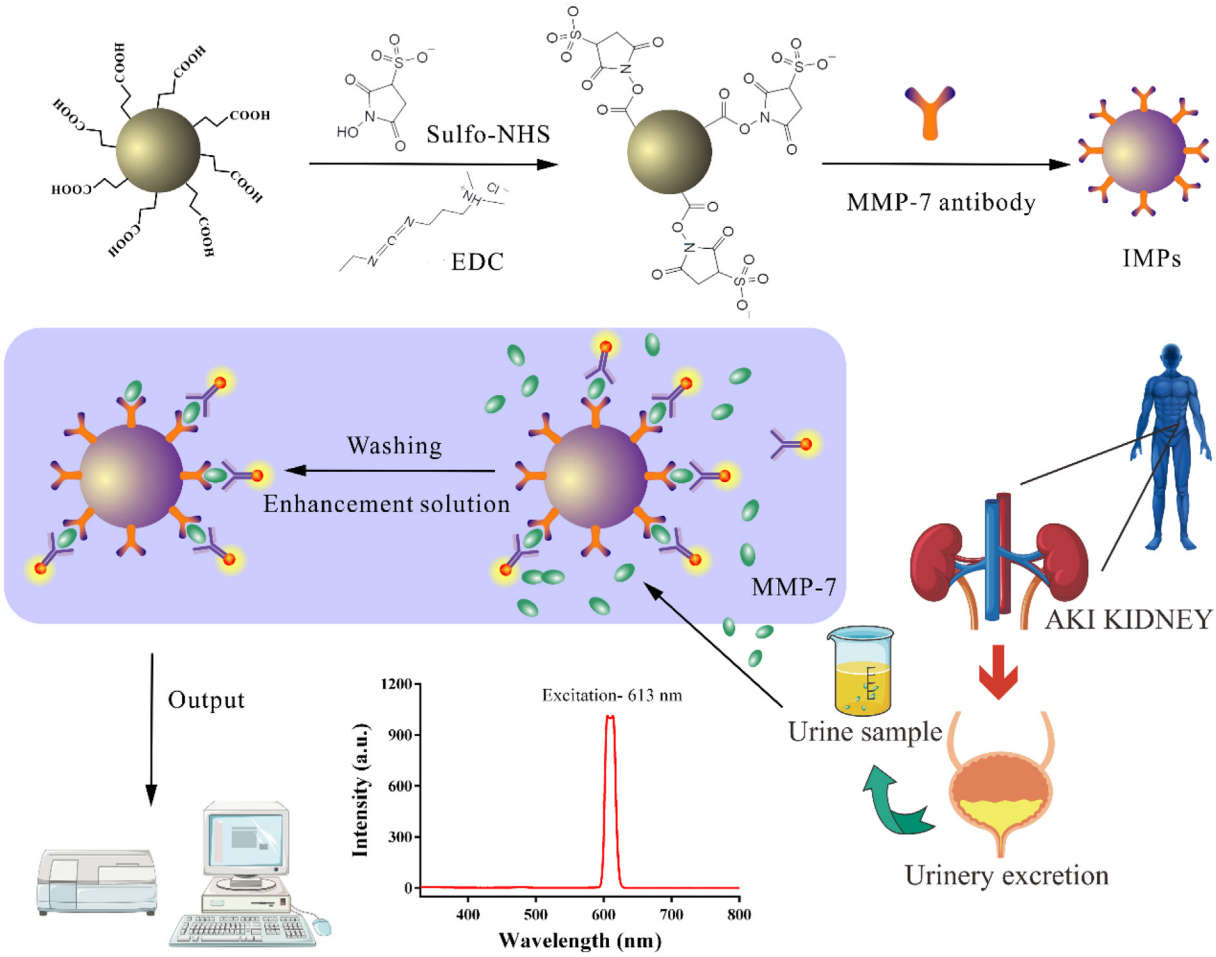
1. Introduction

Acute kidney injury (AKI) is a complex syndrome that significantly affects long-term patient outcome. It occurs in many clinical states, and its clinical manifestations range from a minimal rise in serum creatinine to anuria, which often leads to the need for renal replacement therapy [1,2]. AKI is currently a worldwide public health problem. In China in particular, it poses a burden on the medical system [3], with many cases of underdiagnosis and undertreatment. The non-recognition rate of AKI estimated 74·2% in 2013 in China [3]. Delayed AKI recognition was an independent risk factor for in-hospital mortality. AKI that requires dialysis in critically ill patients is associated with a mortality of 40–70% and the estimated disease prevalence varied from 1 to 25% defined by different definitions of AKI [4,5]. Substantial evidence indicates that AKI increases the risk of advanced composite events that are likely to develop into chronic kidney disease (CKD) [6,7], especially in older patients [8]. Unfortunately, there is currently no effective cure for CKD and the progression to end-stage renal disease, which necessitates

Abbreviations: AKI, Acute kidney injury; CKD, Chronic kidney disease; MMP-7, Matrix metalloproteinase-7; IMPs, Immunomagnetic microparticles; TRF, Time-resolved fluoroimmunoassay; BSA, Bovine serum albumin; MES, 4-morpholineethanesulfonic acid; NHS, N-hydroxysulfosuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; NGAL, Neutrophil gelatinase-associated lipocalin; IL-18, Interleukin 18; KIM-1, Kidney injury molecule 1; Cys C, cystatin C; β_2-MG, β_2-microglobulin; rh, recombinant human

* Corresponding author.
E-mail address: wg@smu.edu.cn (Y. Wu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cca.2018.11.037
Received 19 July 2018; Received in revised form 7 November 2018; Accepted 30 November 2018
Available online 02 December 2018



Scheme 1. Schematic of the noninvasive strategy for determination of the levels of the novel AKI biomarker, uMMP-7.
In vitro diagnosis (IVD) of AKI by measuring the urinary level of MMP-7 using IMPs-TRF to monitor early-onset of AKI. Here is the illustrated synthesis of IMPs and assay procedure of IMPs-TRF.

dialysis or kidney transplantation. Despite recent findings regarding the pathogenesis of AKI and novel therapeutic strategies, the mortality rate in patients remains unacceptably high.

To reduce advanced progression of AKI, early intervention is necessary during the onset of kidney injury. This requires early diagnosis through monitoring of the levels of a battery of reliable biomarkers which are still under research. Though other renal biomarkers are used to help monitoring kidney function including kidney inhury molecule −1 (KIM-1), interleukin 18 (IL-18), cystatin C (Cys C), and β_2-microglobulin (β_2-MG), however, the diagnosis of AKI remains based either on the gold standard, elevated serum creatinine, decreased urine output (> 50%), or the detection of oliguria [2,9]. However, in most cases, increases in serum creatinine do not occur until 48–72 h after the initial insult to the kidney. Therefore, the measurement of serum creatinine is insufficient for predicting the occurrence of AKI or detecting early AKI, as increases in this marker lag far behind renal injury. Moreover, significant renal disease can exist with minimal or no changes in creatinine levels. Although the traditional biomarkers of AKI and CKD such as serum creatinine, blood urea nitrogen, and urinary albumin have been established in clinical practice for decades, they have limitations with respect to the timeliness of diagnosis, sensitivity, and specificity.

Fortunately, several recently identified biomarkers are good indicators of the progression and prognosis of AKI. These include the biomarkers mentioned above and a newly validated and promising biomarker, matrix metalloproteinase-7 (MMP-7) [10]. MMP-7 is a stable and neutral secreted hydrophilic protein with a molecular weight of 28 kDa. It is the smallest matrix metalloproteinase and consists of two domains: a prodomain that is cleaved upon activation and a catalytic domain. It previously gained attention for its role in abnormal tissue remodeling in the early years [11]. MMP-7 is commonly undetected in normal human renal tubular epithelium, but is significantly expressed under certain pathological conditions including polycystic kidney disease, unilateral ureteral obstruction, or during subclinical tubulitis [12,13]. Urinary MMP-7 (uMMP-7) is a biomarker that reflects the activity of intrarenal Wnt/b-catenin, and dependably mirrors its expression in renal parenchyma, particularly in the tubular epithelium. Urinary level of renal MMP-7 protein correlated with renal Wnt/b-catenin activity [14], and uMMP-7 was proposed to represent a noninvasive biomarker of this profibrotic signaling activity in the kidney. Although MMP-7 was a functional biomarker related to CKD [15], increased renal expression of MMP-7 was also related to acute injury, with further increases associating with progression to tubulointerstitial fibrosis [13]. MMP-7 expression also correlated with renal function assessed by glomerular filtration rate (GFR) [16]. We believe that uMMP-7 may serve as an indicator that can help predict the onset of severe AKI following cardiac surgery, and improve risk reclassification compared with using only the clinical model [10]. In a previous study, uMMP-7 predicted the development of severe AKI and was elevated in both subgroups analyzed (those with and without pre-existing CKD). These findings suggest that uMMP-7 can serve as a noninvasive surrogate biomarker of kidney function for predicting the risk of AKI.

Despite its value in predicting the risk of AKI, uMMP-7 has greater utility for the early diagnosis of AKI following major surgery. Further clinical validation of the diagnostic value of MMP-7 in AKI is therefore

required. However, we previously found that the lack of available assays limits its further study and application in both laboratory and clinical practice. Most available quantitative kits are based on traditional enzyme-linked immunosorbent assay (ELISA), and are time-consuming and require dozens of sample dilutions because of inadequate detection ranges. Errors between batches caused by high-ratio dilutions increase as a result. The high ratio of dilutions may affect the results such that measured values will be much higher or lower than actual values. Therefore, these ELISA kits may not be suitable choices for use in clinical practice. In contrast, the time-resolved fluoroimmunoassay (TRF) has low background signals, high sensitivity, and is free of isotopic contamination. These traits are advantageous, and it has been widely used as an analytical tool for clinical diagnosis. In our study, we combined antitarget antibody-conjugated magnetic microparticles as mobile reaction carriers to further improve the sensitivity of this assay. We chose one of the most commonly used lanthanide chelates, Eu^{3+} chelate, as the fluorescent tracer.

In pursuit of an applicable, rapid, and reliable assay for uMMP-7 quantification to resolve the limitation of present analytical technique, we applied an immunomagnetic microparticles-based time-resolved fluoroimmunoassay (IMPs-TRF) to measure uMMP-7 levels in AKI patients (Scheme 1). The analytical performance of the proposed assay was investigated following a method validation protocol and was subsequently validated using clinical samples.

2. Materials and methods

2.1. Reagents and equipment

Bovine serum albumin (BSA), 4-morpholineethanesulfonic acid (MES), proclin-300, Tween-20, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (sulfo-NHS) were from Sigma-Aldrich (St. Louis, MO, USA). Sephadex G-50 was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Magnetic microparticles were from JSR Life Sciences (Tokyo, Japan). The Eu^{3+}-labeling kit and Victor^3 1420 multilabel counter were from PerkinElmer WALLAC (Turku, Finland). iMark™ Microplate Absorbance Reader and ImmunoWash 1575 Microplate Washer were from Bio-Rad Laboratories (Hercules, CA, USA). Two anti-MMP-7 monoclonal antibodies (10–2300 and 10–2299) were from Fitzgerald™ (Fitzgerald Industries International, MA, USA). rhMMP-7 protein (DY907), human Total MMP-7 DUOSET ® ELISA kit, and other kidney functional biomarkers including rhCys C (1196-PI), NGAL (1757-LC), and rhKIM-1 (1750-TM) were from R&D SYSTEMS ® (Cambridge, MA, USA). β_2-microglobulin (β_2-MG) was from Guangzhou Darui Biotechnology Co. LTD (Guangzhou, China).

2.2. Solutions

Buffer solutions used throughout the study were as follows: binding buffer (50 mM MES, pH 5.0), conjugating reagent A (10 mg mL^{-1} EDC, in 50 mM MES, pH 5.0), conjugating reagent B (10 mg mL^{-1} sulfo-NHS, in 50 mM MES, pH 5.0), stock solution (25 mM Tris-base, 150 mM NaCl, 0.05% Tween-20, 5% BSA, 0.05% ProClin300, and 1% trehalose, pH 7.2), elution buffer (50 mM Tris-HCl, 0.9% NaCl, and 0.05% proclin-300, pH 7.8), washing buffer (50 mM Tris-HCl, 0.01% Tween-20, and 150 mM NaCl, pH 7.8), labeling buffer (50 mM Na_2CO_3, pH 9.6), standard buffer (50 mM Tris-base, 150 mM NaCl, 0.1% NaN_3, 0.01% Tween-20, and 7.5% BSA, pH 7.8), assay buffer (50 mM Tris-HCl buffer, 1.5% polyethylene glycol 6000, 0.3 mM BSA, 0.01% Proclin-300, 150 mM NaCl, 0.02% (*w/v*) bovine globulin, and 0.01% Tween-20, pH 7.8), All solutions were freshly prepared before use.

2.3. Synthesis of IMPs

IMPs were synthesized according to a protocol recommended by the manufacturer. First, the carboxyl groups on the surface of magnetic microparticles were activated by EDC and sulfo-NHS before conjugation with anti-MMP-7 monoclonal antibody (10 − 2300). A total of 25 μL of fresh conjugating reagent A and 40 μL of conjugating reagent B (both 10 mg mL^{-1}) were added to 10 mg of carboxyl-modified MPs at a concentration of 100 mg mL^{-1} (10.0×10^9 MPs/mL in sterilized water) in 1 mL of coating buffer, and rotated end-over-end for 30 min at room temperature. Activated carboxyl-modified IMPs were then removed from the magnetic field and washed thrice to remove excess activators. Next, 250 μg of anti-MMP-7 monoclonal antibody (10–2299) in 1 mL of binding buffer was added to the activated MPs and mixed using a vertical mixing apparatus, which gently rotated overnight at 25 °C. After the reaction, the first supernatant was collected, the concentration of residual antibody was measured using a Pierce® Micro BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), and the coupling ratio was calculated. After the samples were washed thrice with stock solution to remove excess antibody, the anti-MMP-7 antibody-conjugated IMPs were blocked with 2 mL of blocking buffer for 4 h after they were successfully synthesized, then washed thrice with stock solution and stored in the same solution at 4 °C until use.

2.4. Preparation of Eu^{3+} chelate-labeled antibody

According to an established approach for the synthesis of Eu^{3+} chelate-labeled antibodies [17], 200 μg of purified antibody (10–2300) was exchanged into chelate-labeling buffer six times using a 10-kD ultrafiltration tube by centrifugation (9500 *g*, 6 min). Next, 40 μg of DTTA-Eu^{3+}chelate was added to the antibody in a total volume of 200 μL. After being fully mixed, the mixture was incubated gently at 25 °C for 24 h, while shielded from light. Free Eu^{3+}-chelates were separated from the tracer by size exclusion chromatography on a Sephadex G-50 column (1.5 cm × 40 cm). After eluting with spent regenerant, about 5 mL of tracer solution was gathered at the sharp protein peak. We then added BSA to the tracer solution to achieve a final concentration of 1 mg mL^{-1} for conservation, and stored the preparation at 4 °C. It was stable for 12 months following the addition of 0.2% BSA as stabilizer.

2.5. Preparation of MMP-7 standards and clinical samples

Serial dilutions of the MMP-7 standard were prepared by diluting recombinant MMP-7 protein in standard buffer to obtain the following standards: 0, 0.1, 0.5, 1, 10, 75, and 150 ng mL^{-1}. Standard solutions were freshly prepared before use. In the present study, a total of 28 urine samples from patients with AKI and 15 urine samples from healthy individuals were obtained from Guilin No.181 Hospital (Guangxi, China), and stored at-20 °C until use. All samples were calibrated by serum creatinine for selection. Creatinine was measured with a Cobas analyzer (Roche Diagnostics) and used to select clinical AKI samples based on Kidney Disease Improving Global Outcomes (KDIGO) guideline. uMMP-7 was also normalized based on the level of urine creatinine (Cr) (uMMP-7/Cr, μg g^{-1}). All urine samples were collected after obtaining verbal informed consent from AKI patients and healthy volunteers. The study was approved by the Ethical Committee of the Science and Technology Department of the Southern Medical University (Guangzhou, China).

2.6. Assay protocol

MMP-7 was measured using a one step double-antibody sandwich method. Samples were analyzed in triplicate. Measurement using the IMPs-TRF was carried out as previously described [18,19]. First, 25 μL of standards or urine samples were added to wells of a 96-well plate. Next, 25 μL of anti-MMP-7 antibody-conjugated IMPs (2.5 μg mL^{-1}) and 50 μL of Eu^{3+}-labeled anti-MMP-7 antibody (2 μg mL^{-1}) were added stepwise, and the mixture was gently incubated for 30 min at

37 °C. After the incubation, the reaction plate was subjected to a magnetic field for several minutes to separate IMPs from the supernatant, with immunocomplex binding on the surface. The supernatant buffer was then removed and the precipitate was washed four times with washing buffer. Finally, 100 μL of enhancement solution was added and incubated for 5 min at 37 °C before measurement with a 1420 Multi-label Counter (Victor3™).

2.7. ELISA

uMMP-7 was quantified using a human Total MMP-7 DUOSET ® ELISA kit (DY907, R&D Systems) according to the manufacturer's instructions. Ten-fold diluted urine samples (100 μL) were added to precoated microplates, and incubated for 1 h. The plates were then washed, 100 μL of biotinylated antibody (1:2000 dilution) was added, and samples were incubated for another 1 h. Then, 100 μL of horse radish peroxidase-labeled streptavidin (1:50 dilution) was used as a tracer. The plates were washed after incubation in all the above steps. Finally, add 100 μL of substrate solution to each well and incubate for 20 min at room temperature and stop by adding 50 μL of stop solution. The absorbance values at 450 nm were then read on a microplate reader (iMark, Bio-Rad Laboratories Inc.).

2.8. Statistical analysis

Standard curves were constructed with the logarithm of the background-subtracted fluorescence value as the dependent variable (Y) against the logarithm of the MMP-7 concentration (ng mL^{-1}) as the independent variable (X), and plotting a line of best fit using Origin Pro7.5 (GE, Piscataway, NJ, USA): log (Y) = A + B × log (X). Signal linearity and correlations were calculated via Pearson's linear regression equation. Linear regression was performed and Pearson's correlation coefficient was determined using SPSS 20.0 (Chicago, IL, USA) to analyze and compare the measured values of urine samples using the proposed method and a commercial ELISA kit. $P < .05$ was considered statistically significant.

3. Results

3.1. Assay validation protocol

Validation of the proposed assay was carried out following Food and Drug Administration guidelines for bioanalytical methods [20]. The analytical performance of the IMPs-TRF was investigated under the requirements of linearity and lower limit of detection (LLOD), accuracy, precision and analytical recovery, selectivity, stability, and validation using clinical samples and method comparisons.

3.2. Linearity and lower limit of detection

Standards diluted in standard buffer as described above (Section 2.5) were applied to construct a calibration curve with the following equation: log(Y) = 3.883 + 0.9226 × log(X). The linearity and LLOD of our assay were then investigated. The calibration curve of the calibrators (Fig. 1) showed there was high linear correlation ($r = 0.9998$) and minimal coefficient variation (< 5%). The LLOQ of IMPs-TRF was defined as the lowset concentration at which MMP-7 can be reliably detected within a proper coefficient of variation (CV% < 20%). The LLOD was defined as the lowest limit of detection and calculated using the following formula [21]: LOB = mean $_{blank}$ + 1.645 × (SD $_{blank}$); LLOD = LOB + 1.645 × (SD $_{low\ concentration\ sample}$). The LLOQ of the IMPs-TRF in determination of uMMP-7 was 0.063 ng mL^{-1} and the LLOD was 0.039 ng mL^{-1}. For this assay, the linearity of detection was 0.063–150 ng mL^{-1} according to the calibration curve of MMP-7 for the IMPs-TRF, which is a large range of coverage for quantification that is appropriate for the biological levels of MMP-7 in AKI patients. The

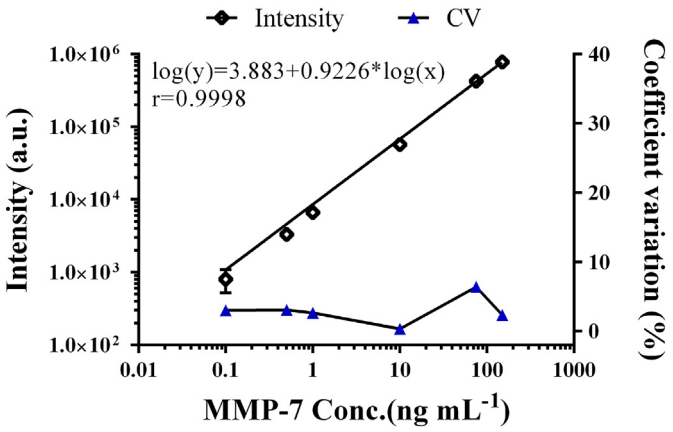


Fig. 1. Calibration curve of MMP-7 with the IMPs-TRF.

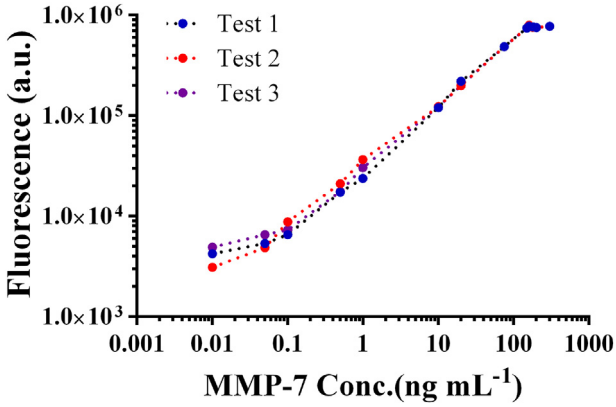


Fig. 2. The hook effect of uMMP-7 detected by the IMPs-TRF.

high-dose hook effect for the IMPs-TRF was also discussed using anchoring points in the asymptotic high concentration of MMP-7 (Fig. 2). When the concentration of MMP-7 was higher than 150 ng mL^{-1}, fluorescence intensity stopped increasing, indicating that levels higher than this concentration could impede the immunoreaction, and that urine samples with extremely high levels of MMP-7 should be diluted so as to be within the appropriate concentration range. Fortunately, the levels of MMP-7 are within the detection range in most cases.

3.3. Accuracy, precision and analytical recovery of the IMPs-TRF

The accuracy of the test was assessed after optimal reaction conditions were established. Three blank urine samples with MMP-7 added at low, medium, and high concentrations were analyzed in five duplicates for assessment of intra-assay precision, and 20 duplicates were analyzed for interassay precision. As shown in Table 1, the coefficient variation of the intra- and interassay analyses were low (≤ 5.17%). Neither imprecisions were high (≥10%). To investigate recovery, we analyzed three urine samples spiked with MMP-7 (Low: 1 ng mL^{-1}, Medium:

Table 1
Precision of the IMPs-TRF in determination of the level of uMMP-7.

Samples (ng mL^{-1})		Mean	SD	CV (%)
Intra-assay precision ($n = 5$)	5	4.97	0.13	2.58
	10	10.84	0.33	3.04
	20	19.76	0.79	4.01
Inter-assay precision ($n = 20$)	5	5.00	0.15	3.01
	10	10.84	0.57	5.17
	20	19.92	1.00	5.00

SD: standard deviation; CV: coefficient variation.

Table 2
Analytical recovery of urine samples spiked with MMP-7 (n = 5).

Samples (ng mL^{-1})	Expected	Observed	Recovery (%)
Low	1	1.05 ± 0.05	105.60 ± 5.95
Medium	10	10.32 ± 0.67	103.18 ± 6.74
High	150	148.60 ± 3.60	99.06 ± 2.40

10 ng mL^{-1}, High: 150 ng mL^{-1}) in duplicate five times using the IMPs-TRF. 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 99.06%–105.60% (Table 2).

3.4. Selectivity and stability analyses

To assess whether the presence of other kidney functional biomarkers affected the assay, the crossreactivities of rhMMP-7, rhCys C, NGAL, rhKIM-1, and β_2-MG were evaluated using the IMPs-TRF. All of these proteins were diluted in assay buffer at high biological concentrations (Cys C, 500 ng mL^{-1}, NGAL, 1000 ng mL^{-1}, KIM-1, 1000 ng mL^{-1}, and β_2-MG, 10 μg mL^{-1}). Crossreactivities of other MMPs, including MMP-2, MMP-3, MMP-9 and MMP-14, which increased in urine when kidney diseases occur were also evaluated at high concentration (1000 ng mL^{-1}). The results of the selectivity test are shown in Table 4, and indicate that the antibody applied in our proposed assay is highly specific to the MMP-7 antigen, and has minimal crossreactivity with other relevant kidney functional biomarkers and other renal related MMPs. The relative signal intensities of NGAL, rhKIM-1, and β_2-MG were very low (< 0.5%), as shown in Fig. 3. The high level of Cys C caused a slightly higher relative signal intensity (< 2.2%), although it remained within an acceptable range. The crossreactivities of MMPs was acceptable (< 5.35%). Such interactions did not affect the calibration curve or the limit of detection of the assay. The crossreactivities of these high-level urinary biomarkers were minimal.

Regarding the stability analysis, the low and high concentrations of the spiked samples diluted in standard buffer were 10 ng mL^{-1} and 100 ng mL^{-1}, respectively. The samples were stored at −20 °C and 37 °C for 24 h, and thawed unassisted at room temperature. All the detected signals were above 85% of the signals of original test before the treatment. Additionally, three freeze/thaw cycles at −20 °C and a long-term stability experiment (20 d) were performed. This analytical reagent was stable for long-term storage at −20 °C, and its repeated freezing and thawing should be avoided (Table 3).

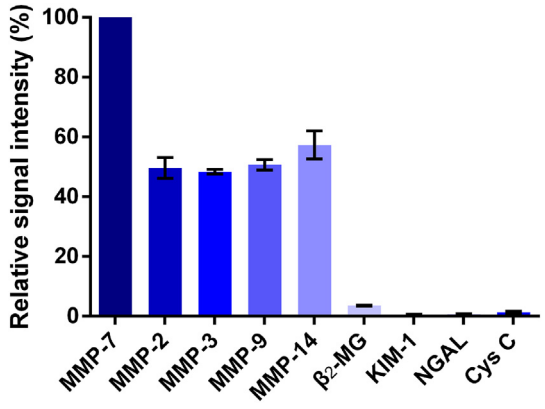


Fig. 3. Selectivity for the measurement of uMMP-7 using the IMPs-TRF.

Table 3
Selectivity for the measurement of uMMP-7 levels using the IMPs-TRF.

Analytes	Concentration (ng mL^{-1})	Detected (ng mL^{-1})	Cross-reactivity (%)
KIM-1	1000	0.031	0.003
NGAL	1000	0.051	0.005
Cys C	500	0.111	0.022
β_2-MG	10,000	0.295	0.003
MMP-2	1000	4.526	0.453
MMP-3	1000	4.393	0.439
MMP-9	1000	4.644	0.464
MMP-14	1000	5.535	0.535
MMP-7	10	9.500	95.00

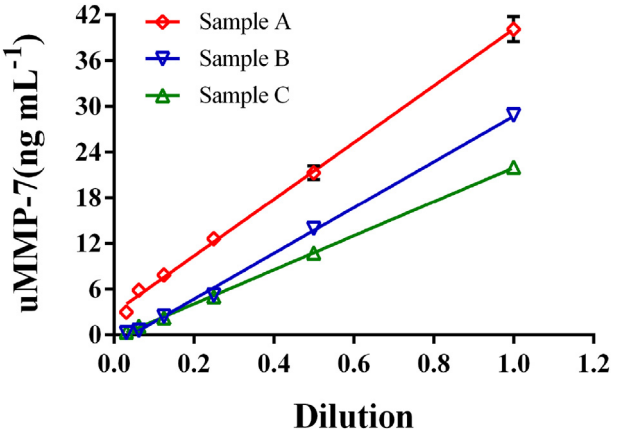


Fig. 4. Dilution curve for the IMPs-TRF based on three samples.

3.5. Validation using clinical sample and method comparison

Urine samples of three concentrations (41.2, 29.3 and 22.8 ng mL^{-1}) were diluted in doubled dilutions and detected using IMPs-TRF. The linearity of dilution curves for all three samples were also good, showing little variation in the measured concentrations after correcting for sample dilution factors (Fig. 4). 28 clinical urine samples of AKI patients were simultaneously tested using IMPs-TRF and DUOSET@ ELISA kit (Fig. 5). All testing was performed in triplicate. We performed parallel comparison of test results obtained from two analytical methods, IMPs-TRF and ELISA, and the results presented good correlation both in comparison of uMMP-7 level (ng mL^{-1}) and uMMP-7 / Cr (μg g^{-1}) (Fig. 5A and C). The coefficient of correlation r was 0.9541 and 0.9595, respectively. Graphic comparison results were also plotted in Fig. 5B and D. The level of MMP-7/Cr from most AKI samples was much higher than that from non-AKI healthy controls (Fig. 6).

4. Discussions

We previously found that the measured values of uMMP-7 in high level samples were inconsistent with actual values. More specifically, they were higher than actual values when the samples were diluted at a high ratio before measurement. Aiming to resolve this issue, we devised a more rapid and precise quantitative assay. In the present study, the IMPs-TRF improved upon the traditional TRF -by integrating IMPs, which develops a semi-homogeneous reaction that the analyte can react with the carrier IMPs under uniform distribution in liquid phase. Based on IMPs, immunocomplexes form rapidly in the mobile reacting phase, and are then easily autoseparated from excess analyte in a magnetic field [22–24]. This is followed by amplification of the fluorescence signal through an enhancement process which enables a shorter reaction time and extremely high signal to remove background interference. Comparison of the assay with two commercial ELISA kits from R&D SYSTEMS and TRACE is shown in Table 4. Regarding reaction time, the

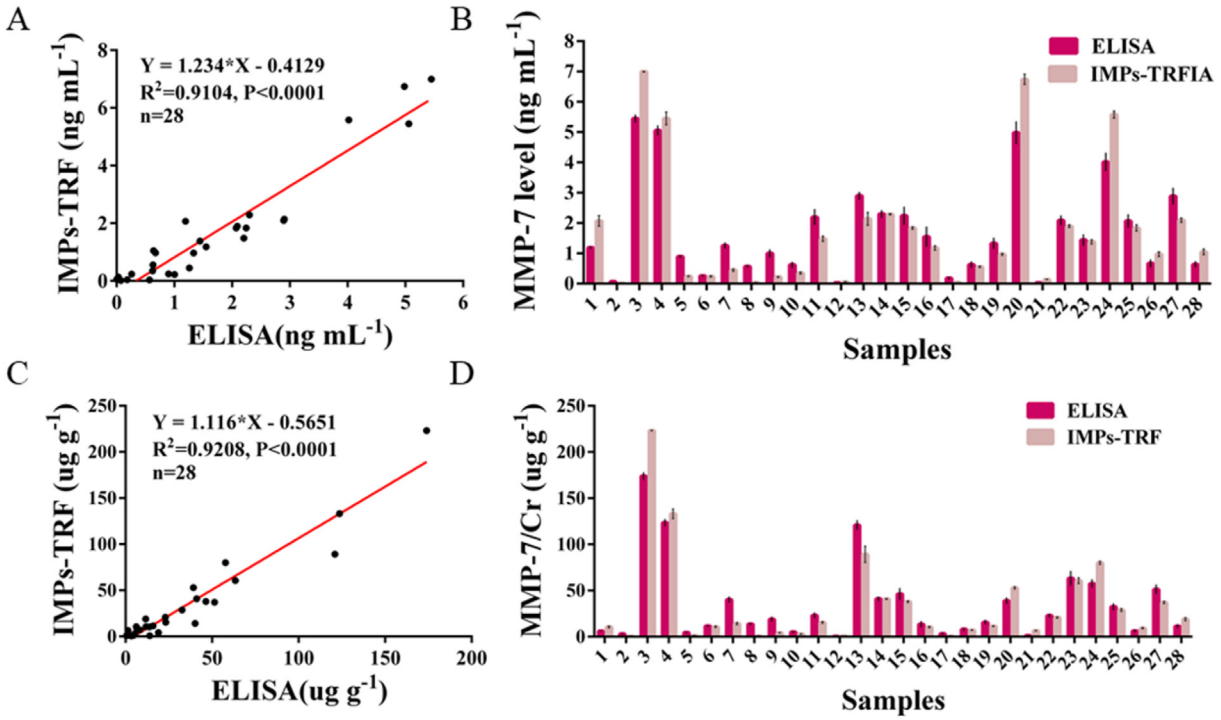


Fig. 5. Regression analysis of the values of uMMP-7 between the IMPs-TRF and ELISA.

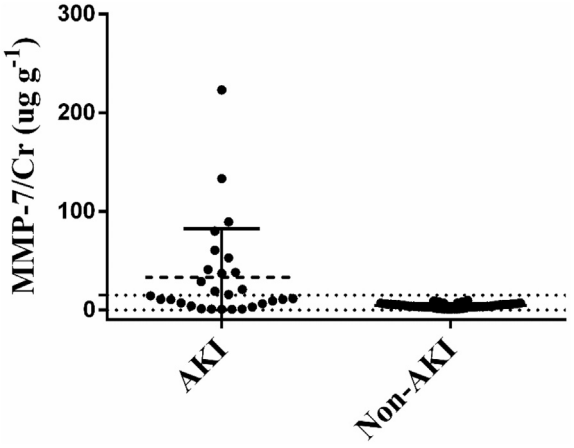


Fig. 6. MMP-7/Cr level between AKI and non-AKI samples.

IMPs-TRF is clearly superior to ELISA. Measurement of MMP-7 levels using a TRACE technology-based BRAHMS KRYPTOR analyzer (Thermo Fisher Scientific) reduces reaction time compared with ELISA, but the sample materials for analysis are limited to serum or plasma and it requires more effort for improvement in testing of urine samples. Compared with ELISA and the TRACE method, the proposed IMPs-TRF has a much wider linear range of detection and lower sensitivity. For sample dilution, both ELISA kits required 10 to dozens of dilutions to meet the quantitative limit, while the IMPs-TRF is free from high-ratio dilution. That's because the fluorescent characteristic of the Eu^{3+} chelate which has a high signal-to-noise ratio, allows for both a relatively wide linear range of detection and high anti-interference capacity. Easy automation is another major advantage of the IMPs-TRF, which reduces error caused by manual operation. In general, the proposed IMPs-TRF has satisfactory analytical performance and surpasses that of the available assays described above.

When patients with cardiovascular disease increase remarkably, the incidence of AKI is unfavourable. In general, AKI is associated with high rates of mortality and poor treatment options that are primarily because of the narrow therapeutic window of AKI. This makes it difficult to evaluate and monitor the phase and development of AKI. With a narrow therapeutic widow and limited treatment time, medical interventions for AKI patients are only effective at the early onset. Although AKI is preventable, the implementation of current preventive strategies is suboptimal. The fundamental principle of prevention of AKI is to treat the cause or triger. When prerenal factors contribute, they should be identified, haemodynamic resuscitation quickly begun, and intravascular volume maintained or rapidly restored [25]. According to KDIGO guideline, acute and chronic kidney diseases are defined and staged based primarily on GFR as a measure of kidney function, and albuminuria as a marker of kidney damage [26]. GFR is assessed from clearance measurements or estimated from serum levels of endogenous filtration markers such as creatinine or cystatin C [9,27]. These

Table 4
Comparison of other analytical methods available for the measurement of MMP-7.

Reagents	Linearity (ng mL^{-1})	Reaction time	Sample volume	LLOD	Inaccuracy	Dilution
DuoSet ELISA Kit	0.0078–2	5 h	100 μl	N/A	< 7.22%	> 1/20
Quantikine ELISA Kit	0.2–10	4.5 h	50 μl	0.094 ng mL^{-1}	< 5.1%	> 1/5
TRACE [33]	0.5–40	19–59 min	50 μl	N/A	N/A	N/A
IMPs-TRF	0.063–150	30 min	25 μl	0.039 ng mL^{-1}	< 5.17%	None

TRACE: time-resolved amplified cryptate emission.
LLOD: lower limit of detection; N/A: not available.

indicators are reliable but lag far behind the loss of kidney function. In recent years, numerous studies aimed to identify and validate potential new biomarkers related to AKI aside from the level of serum creatinine, which remains the gold standard criterion for diagnosis but has limitations. It is unable to aid in the diagnosis of acute renal injury during the onset period or differentiate among its various causes. Among these biomarkers, Cystatin C (which is considered to reflect disorders of tubular reabsorption), NGAL, IL-18 and KIM-1 are the most promising tubular biomarkers of tubular injury in AKI with high performance that reflect either renal tubular epithelial cell damage, reactivity of tissue damage, or correlate with inflammation [28–30]. The diagnostic value of these markers alone may be insufficient. In many cases, different combinations of these biomarkers assist in the diagnosis and prognosis of different renal diseases. Regarding the different stages of AKI, different combinations of diagnostic biomarkers are relevant for prognosis and treatment. Regarding the early diagnosis of AKI, most studies focused on NGAL, KIM-1, and IL-18, alone or in combination [31–33]. uMMP-7 is newly validated as being highly useful in predicting the risk of AKI after major surgery and may represent a promising noninvasive surrogate biomarker in the early diagnosis of AKI and for the monitoring of renal function. uMMP-7 is also associated with other renal diseases such as the progression of CKD, renal cell carcinoma, and tubulointerstitial fibrosis. The assay proposed herein may serve as a powerful tool for clinical practitioners making further research on uMMP-7 more convenient.

In the future, noninvasive methods such as urine testing may become mainstream, and the combination of uMMP-7 and other renal biomarkers may have greater diagnostic value for acute renal diseases. Therefore, our further studies will focus on improving analytical methods for rapid diagnosis of kidney diseases using multiple renal biomarkers in urine samples. Based on our previous research on multi-labels detection [34,35], to achieve multiple analyses in a single test, we envision the development of dual-labeling in quantification of renal biomarkers by taking advantage of IMPs-TRF that no intersection of emission spectrum between different lanthanide fluorescent tracers such as europium and samarium.

5. Conclusions

In the present study, we introduced a sensitive analytical method to quantify the levels of uMMP-7 in clinical samples. The IMPs-TRF method has superior analytical performance over traditional ELISA in many respects, most notably with its wide quantitative range (0.063–150 ng mL^{-1}) and short reaction time (30 min). We believe the proposed automated assay can be used as an efficient tool, in conjunction with other diagnostic indicators of AKI, to achieve effective early diagnosis and early intervention in clinical practice.

Conflict of interest

None.

Acknowledgments

The work is supported by the National Natural Science Foundation of China [Grant No. 81702072], the Science and Technology Plan Project of Guangzhou [201508020260] and the Postdoctoral Science Foundation of China [Grant No. 2016M600662].

References

[1] J. Malyszko, Biomarkers of acute kidney injury in different clinical settings: a time to change the paradigm, Kidney Blood Press. Res. 33 (5) (2010) 368–382.
[2] R.L. Mehta, Acute renal failure definitions and classification: time for change? J. Am. Soc. Nephrol. 14 (8) (2003) 2178–2187.
[3] L. Yang, G. Xing, L. Wang, Y. Wu, S. Li, G. Xu, Q. He, J. Chen, M. Chen, X. Liu, Z. Zhu, L. Yang, X. Lian, F. Ding, et al., Acute kidney injury in China: a cross-sectional survey, Lancet (London, England) 386 (10002) (2015) 1465–1471.
[4] VNARFT Network, P.M. Palevsky, J.H. Zhang, T.Z. O'Connor, G.M. Chertow, S.T. Crowley, D. Choudhury, K. Finkel, J.A. Kellum, E. Paganini, R.M.H. Schein, M.W. Smith, K.M. Swanson, B.T. Thompson, A. Vijayan, S. Watnick, R.A. Star, P. Peduzzi, Intensity of renal support in critically ill patients with acute kidney injury, N. Engl. J. Med. 359 (1) (2008) 7–20.
[5] S. Uchino, J.A. Kellum, R. Bellomo, G.S. Doig, H. Morimatsu, S. Morgera, M. Schetz, I. Tan, C. Bouman, E. Macedo, N. Gibney, A. Tolwani, C. Ronco, Acute renal failure in critically ill patients: a multinational, multicenter study, JAMA 294 (7) (2005) 813–818.
[6] L.S. Chawla, P.W. Eggers, R.A. Star, P.L. Kimmel, Acute kidney injury and chronic kidney disease as interconnected syndromes, N. Engl. J. Med. 371 (1) (2014) 58–66.
[7] J. Vanmassenhove, J. Kielstein, A. Jörres, W.V. Biesen, Management of patients at risk of acute kidney injury, Lancet 389 (10084) (2017) 2139–2151.
[8] D. Neves, A. Belo, A.F. Damásio, J. Carvalho, A.R. Santos, B. Piçarra, J. Aguiar, Acute kidney injury in acute coronary syndromes - an important multifactorial consequence, Rev. Port. Cardiol. 35 (7-8) (2016) 415–421.
[9] L.A. Stevens, J. Coresh, T. Greene, A.S. Levey, Assessing kidney function–measured and estimated glomerular filtration rate, N. Engl. J. Med. 354 (23) (2006) 2473–2483.
[10] X. Yang, C. Chen, S. Teng, X. Fu, Y. Zha, H. Liu, L. Wang, J. Tian, X. Zhang, Y. Liu, J. Nie, F.F. Hou, Urinary matrix metalloproteinase-7 predicts severe AKI and poor outcomes after cardiac surgery, J. Am. Soc. Nephrol. 28 (11) (2017) 3373–3382.
[11] H. Nagase, J.F. Woessner Jr., Matrix metalloproteinases, J. Biol. Chem. 274 (31) (1999) 21491–21494.
[12] P. Hirt-Minkowski, H.-P. Marti, G. Hönger, D. Grandgirard, S.L. Leib, P. Amico, S. Schaub, Correlation of serum and urinary matrix metalloproteases/tissue inhibitors of metalloproteases with subclinical allograft fibrosis in renal transplantation, Transpl. Immunol. 30 (1) (2014) 1–6.
[13] K. Surendran, T.C. Simon, H. Liapis, J.K. McGuire, Matrilysin (MMP-7) expression in renal tubular damage: association with Wnt4, Kidney Int. 65 (6) (2004) 2212–2222.
[14] W. He, R.J. Tan, Y. Li, D. Wang, J. Nie, F.F. Hou, Y. Liu, Matrix metalloproteinase-7 as a surrogate marker predicts renal Wnt/beta-catenin activity in CKD, J. Am. Soc. Nephrol. 23 (2) (2012) 294–304.
[15] D. Zhou, Y. Tian, L. Sun, L. Zhou, L. Xiao, R.J. Tan, J. Tian, H. Fu, F.F. Hou, Y. Liu, Matrix metalloproteinase-7 is a urinary biomarker and pathogenic mediator of kidney fibrosis, J. Am. Soc. Nephrol. 28 (2) (2017) 598–611.
[16] S.M.T.C.R. Ban, B. Franjic, B.A. Brooks, D. Celermajer, S.V.M.D.K. Yue, 2.5-Serum MMP-7 is increased in diabetic renal disease anddiabetic diastolic dysfunction, Diabetes Research and Clinical Practice, 87 2010, pp. 335–341.
[17] J.Y. Hou, T.C. Liu, G.F. Lin, Z.X. Li, L.P. Zou, M. Li, Y.S. Wu, Development of an immunomagnetic bead-based time-resolved fluorescence immunoassay for rapid determination of levels of carcinoembryonic antigen in human serum, Anal. Chim. Acta 734 (2012) 93–98.
[18] Z.-Q. Ren, T.-C. Liu, S.-H. Zhuang, G.-F. Lin, J.-Y. Hou, Y.-S. Wu, Establishment of magnetic microparticles-assisted time-resolved fluoroimmunoassay for determinating biomarker models in human serum, PLoS One 10 (6) (2015) e0130481.
[19] J.-Y. Hou, T.-C. Liu, G.-F. Lin, Z.-X. Li, L.-P. Zou, M. Li, Y.-S. Wu, Development of an immunomagnetic bead-based time-resolved fluorescence immunoassay for rapid determination of levels of carcinoembryonic antigen in human serum, Anal. Chim. Acta 734 (2012) 93–98.
[20] Guidance for Industry Bioanalytical Method Validation, U.S. Department ofHealth and Human Services, Food and Drug Administration Center for DrugEvaluation and Research (CDER) Center for Veterinary Medicine (CVM), (2001).
[21] D.A. Armbruster, T. Pry, Limit of blank, limit of detection and limit of quantitation, Clin. Biochem. Rev. 29 (Suppl. 1) (2008) S49–S52.
[22] G. Lin, T. Liu, J. Hou, Z. Ren, J. Zhou, Q. Liang, Z. Chen, W. Dong, Y. Wu, A magnetic nanoparticle-based time-resolved fluoroimmunoassay for determination of the cytokeratin 19 fragment in human serum, J. Fluoresc. 25 (2) (2015) 361–367.
[23] Z.Q. Ren, T.C. Liu, S.H. Zhuang, G.F. Lin, J.Y. Hou, Y.S. Wu, Establishment of magnetic microparticles-assisted time-resolved fluoroimmunoassay for determinating biomarker models in human serum, PLoS One 10 (6) (2015) e0130481.
[24] J.Y. Hou, T.C. Liu, Z.Q. Ren, M.J. Chen, G.F. Lin, Y.S. Wu, Magnetic particle-based time-resolved fluoroimmunoassay for the simultaneous determination of alpha-fetoprotein and the free beta-subunit of human chorionic gonadotropin, Analyst 138 (13) (2013) 3697–3704.
[25] R. Bellomo, J.A. Kellum, C. Ronco, Acute kidney injury, Lancet 380 (9843) (2012) 756–766.
[26] A.S. Levey, C. Becker, L.A. Inker, Glomerular filtration rate and albuminuria for detection and staging of acute and chronic kidney disease in adults: a systematic review, JAMA 313 (8) (2015) 837–846.
[27] J. Bjork, S.E. Back, N. Ebert, M. Evans, A. Grubb, M. Hansson, I. Jones, E.J. Lamb, P. Martus, E. Schaeffner, P. Sjostrom, U. Nyman, GFR estimation based on standardized creatinine and cystatin C: a European multicenter analysis in older adults, Clin. Chem. Lab. Med. 56 (3) (2018) 422–435.
[28] I.E. Hall, S.G. Yarlagadda, S.G. Coca, Z. Wang, M. Doshi, P. Devarajan, W.K. Han, R.J. Marcus, C.R. Parikh, IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation, J. Am. Soc. Nephrol. 21 (1) (2010) 189–197.
[29] L. Hoskova, J. Franekova, I. Malek, J. Kautzner, O. Szarszoi, A. Jabor, M. Pindak, O. Viklicky, V. Melenovsky, Comparison of Cystatin C and NGAL in early diagnosis of acute kidney injury after heart transplantation, Ann. Transplant. 21 (2016) 329–345.
[30] C. Francoz, M.K. Nadim, F. Durand, Kidney biomarkers in cirrhosis, J. Hepatol. 65 (4) (2016) 809–824.
[31] J. Ho, N. Tangri, P. Komenda, A. Kaushal, M. Sood, R. Brar, K. Gill, S. Walker,

K. MacDonald, B.M. Hiebert, R.C. Arora, C. Rigatto, Urinary, plasma, and serum biomarkers' utility for predicting acute kidney injury associated with cardiac surgery in adults: a meta-analysis, Am. J. Kidney Dis. 66 (6) (2015) 993–1005.

[32] M. Nejat, J.W. Pickering, P. Devarajan, J.V. Bonventre, C.L. Edelstein, R.J. Walker, Z.H. Endre, Some biomarkers of acute kidney injury are increased in pre-renal acute injury, Kidney Int. 81 (12) (2012) 1254–1262.

[33] M. Sokolski, R. Zymlinski, J. Biegus, P. Siwolowski, S. Nawrocka-Millward, J. Todd, M.R. Yerramilli, J. Estis, E.A. Jankowska, W. Banasiak, P. Ponikowski, Urinary levels of novel kidney biomarkers and risk of true worsening renal function and mortality in patients with acute heart failure, Eur. J. Heart Fail. 19 (6) (2017) 760–767.

[34] G. Lin, H. Zhao, T. Liu, J. Hou, Z. Ren, W. Huang, W. Dong, Y. Wu, Simultaneous determination of the cytokeratin 19 fragment and carcinoembryonic antigen in human serum by magnetic nanoparticle-based dual-label time-resolved fluoroimmunoassay, RSC Adv. 4 (98) (2014) 55229–55236.

[35] J.W. Zhou, L.M. Lei, Q.N. Liang, T.C. Liu, G.F. Lin, Z.N. Dong, R.L. Liang, Z.H. Chen, Y.S. Wu, Dual-labeled time-resolved immunofluorometric assay for the determination of IgM antibodies to rubella virus and cytomegalovirus in human serum, Clin. Biochem. 48 (9) (2015) 603–608.