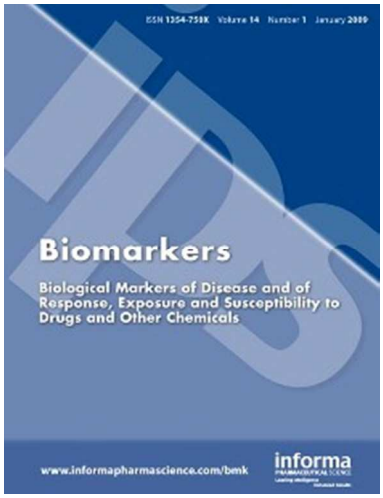

A Miniaturized Immunoassay Platform to Measure Neutrophil Gelatinase-Associated Lipocalin (NGAL) for Diagnosis of Acute Kidney Injury

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A Miniaturized Immunoassay Platform to Measure Neutrophil Gelatinase-Associated Lipocalin (NGAL) for Diagnosis of Acute Kidney Injury

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ABSTRACT

In this study, we describe the development and evaluation of a slide-based immunoassay platform for the detection of Neutrophil Gelatinase Associated-Lipocalin (NGAL) in plasma and urine samples. The capture NGAL antibody was immobilized onto a microscope slide before an analysis of NGAL based on a sandwich immunoassay was further carried out. This assay system exhibited linearity between 50 to 1,000 ng/ml of NGAL. The coefficients of variability (CVs) indicated good reproducibility and repeatability of the system. The levels of plasma NGAL measured by the slide-based system were highly correlated with those of ELISA, while this system over-predicted urine NGAL.

Keywords: Acute Kidney Injury, Neutrophil Gelatinase-associated Lipocalin, slide-based immunoassay

INTRODUCTION

Acute kidney injury (AKI) is characterized by a deterioration of renal function over a period of hours to days (Han et al, 2004). The mortality and morbidity rate of hospitalized patients with AKI has increased in recent years and become a worldwide public health problem. Current diagnosis of AKI relies on the evaluation of the levels of serum creatinine which is insensitive, non-specific, and prone to interference from external factors (Naud et al, 2008). Moreover, its concentration does not change until 24 – 48 hours after significant kidney damage. Therefore, novel biomarkers for kidney injury have played a more important role in early identification of AKI (Vaidya et al, 2008). Neutrophil gelatinase–associated lipocalin (NGAL) or Lipocalin-2 (LCN2) has been reported as one of the most promising biomarkers for the prediction of AKI. NGAL is a 25 kDa protein covalently bound to gelatinase in specific granules of neutrophil (Devarajan et al, 2007). Normally, the expression of NGAL is very low in several human tissues such as kidneys, lungs, stomachs, etc. Nevertheless, the renal expression of NGAL is markedly increased after ischemic or nephrotoxic injury, and NGAL is released into both urine and blood stream (Cowland et al, 1997).

Quantitative measurements of NGAL are typically performed using the enzyme-linked immunosorbent assay (ELISA) in a well-based platform. This technique is based on an antigen and its corresponding antibody to recognize each other and form a complex due to their specific affinity. Although ELISA provides precise and accurate results, it requires more than 4 hours of assay time and a large amount of reagent, making this technique expensive and cumbersome for routine use in clinical practice. Recently, two commercially available NGAL test kits have been developed. Architect analyzer (Abbott Diagnostics) determines NGAL in urine, while Alere's Triage device, a point-of-care immunoassay system, measures

plasma NGAL (Devarajan, 2008). A high degree of variability (up to 35%) was observed around the cut-off for a reference value of AKI (below 130 ng/ml) when Triage device was used (Cavalier et al, 2011). This would easily cause problems in the interpretation of results. In comparison, Architect analyzer provides superior analytical accuracy and performance than Triage device. However, the analysis of urine NGAL using the Architect platform requires specialized and expensive equipment which is not available in most laboratories due to large investment (at least $200,000 for the analyzer alone). Thus, a simple and inexpensive system for the determination of NGAL is desired.

In this work, a miniaturized immunoassay system in a planar platform was developed to address the problem of high reagent consumption. The binding of antigen-antibody is believed to be more efficient in microscale as a result of high surface area to volume ratio (Wingren et al, 2009). The new system, utilizing a non-competitive sandwich format with fluorescent signal detection, was used to measure plasma and urine NGAL levels in healthy controls and AKI patients. The results were validated against those of the conventional method (ELISA) to assess the performance of the new assay platform.

MATERIALS AND METHODS

2.1 Fabrication of an antibody-immobilized slide

A microscope glass slide was cleaned with freshly prepared Piranha solution (30% H_2O_2: 70% H_2SO_4) for 30 minutes at room temperature, washed with deionized water and dried under nitrogen flow. To modify the surface, the slide was soaked with 2% v/v of 3-aminopropyltriethoxy silane (APTES) in acetone for 2 minutes. After removal of the solution, the slide was rinsed several times with acetone and water to remove unbound silane and dried. The dried slide was immersed in succinic anhydride pH 6.0 for 2 hours at room temperature to generate carboxyl group on the surface. The slide was rinsed with phosphate citrate buffer pH 4.6 and dried under nitrogen flow.

One-half microliter of a mixture containing 400 µg/ml of ethyl(dimethylaminpropyl) carbodiimide (EDC), 600 µg/ml of n-hydroxysuccinimide (NHS) and 8 µg/ml of anti-human NGAL (R&D Systems, Minneapolis, USA) in phosphate citrate buffer, pH 4.6, was spotted on the modified surface using a manual pipette following the design of the glass slide, as illustrated in Figure 1A. The capture antibody was allowed to immobilize on to the surface for 1 hour at room temperature. The reaction was terminated with Tris-HCl buffer for 2 minutes, washed with phosphate buffer saline (PBS), and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C to block any non-specific protein adsorption.

2.2 ***Immunoassay***

The immunoassays were conducted following the procedure for sandwich-type assays. A sample was added to an antibody immobilized glass slide and incubated for 1 hour, followed by rinsing with PBS. Subsequently, 0.4 µg/ml of anti-human NGAL biotinylated antibody (R&D Systems, Minneapolis, USA) was added and incubated for 1 hour. The slide was

washed with PBS and the biotinylated antibody diluted at 1:3000 was hybridized with Northernlight™ 493-strepavidin (R&D Systems, Minneapolis, USA) and incubated for 30 minutes at room temperature in the dark. The glass slide was imaged with Typhoon Trio scanner (Amersham Pharmacia Biotech, USA) using 488 nm excitation with 520 BP emission filters. The intensity of fluorescence was analyzed with ImageQuant™ software (Amersham Pharmacia Biotech, USA).

2.3 *Enzyme-Linked Immunosorbent Assay*

A human NGAL ELISA kit (R&D Systems, Minneapolis, USA) was used to detect human NGAL in plasma and urine samples and was performed following the manufacturer's instruction. Based on the measurement interval of ELISA, the samples of the healthy controls were diluted 100 fold, while the samples of the AKI patients were diluted 800 fold. Briefly, 100 µl/well of the assay diluent and 50 µl/well of the sample were added to the antibody-coated microwell and incubated for 2 hours at 2 – 8°C. The plate was washed four times. A 200 µl/well of peroxidase conjugated anti-Human NGAL antibody was subsequently added and incubated for 2 hours at 2 - 8°C. The washing step was repeated. Another 200 µl/well of a substrate solution for peroxidase (3,3',5,5'-tetramethyl benzidine and H_2O_2) was added before being incubated for 30 minutes at room temperature. The reaction was stopped by an addition of 50 µl/well of 1 M H_2SO_4. The plate was read using a wavelength of 450 nm in a microplate reader (Tecan Group, Switzerland).

2.4 Plasma and urine samples

Blood and urine samples were obtained from 20 patients diagnosed with AKI at the King Chulalongkorn Memorial Hospital and from 20 healthy volunteers who gave their informed consent. The information of the patients, such as age, gender, severity and renal replacement therapy (RRT) requirement, is reported in Table 3. The blood samples were collected using

heparin tubes and centrifuged at 2,500 rpm for 5 minutes at room temperature. The supernatant in equal volume was stored at -20°C until use. For urine samples, midstream urine was collected in a sterile container. The supernatant was aliquoted after centrifugation at 3,000 rpm for 5 minutes at 4°C and rapidly stored at -80 ºC until further analysis.

2.5 Data Analysis and Statistics

The precision or reproducibility of the assay was expressed in terms of coefficient of variability (CV) which was calculated as shown in Eq.1.

$$\%CV = \frac{s\tan dard\ deviation}{mean} \times 100 \quad (1)$$

The error of the measured concentration from the actual concentration was calculated using Eq.2.

$$\%error = \frac{|measured\ conc. - actual\ conc.|}{actual\ conc.} \times 100 \quad (2)$$

Data are presented as mean ± SD. An unpaired t-test was used for data analyses. A statistically significant difference was defined at the 95% confidence level.

RESULTS AND DISCUSSION

3.1 *Design of an antibody immobilized slide and calibration curve*

A schematic diagram of our antibody-immobilized glass slide is shown in Figure 1A. The glass slide was designed to accommodate 90 spots (6 × 15 arrays) of immobilized capture anti-NGAL antibody, allowing for 90 measurements. The diameter of each spot was approximately 1.2 ± 0.1 mm with 5 mm spacing. In a typical experiment, spots A1 - A6 served as negative control for a buffer solution without NGAL protein to check for non-specific binding and false positive. The spots on row 7 were regarded as "blank", because of absent capture antibody, to determine the background of this assay system. The rest of the antibody spots were used for protein standards and test samples.

To construct a calibration curve, the recombinant NGAL was diluted between 10 – 1000 ng/ml. The detail of the experimental design is shown in Supplementary A. The measurement of each concentration was repeated 6 times. Figure 1B shows a fluorescence image of the slide used to generate the calibration curve. Clearly, The intensity of fluorescence was proportional to the concentration of NGAL. The concentration of NGAL at 1000 ng/ml yielded the maximum fluorescence intensity, as shown in bright green color. The fluorescence intensities of blank and negative control were considered negligible, indicating no non-specific binding outside the immobilized antibody location.

The fluorescence intensity of each spot was corrected using background subtraction to generate a calibration curve. The fluorescence intensity was shown to be linearly proportional to NGAL concentrations between 50 to 1000 ng/ml with a correlation coefficient (R^2) of 0.9903 (Figure2). The limit of detection was estimated to be 25 ng/ml which corresponded to a signal 3 times the background noise.

3.2 *Assay Precision*

The precision or reproducibility of assay is expressed in terms of coefficient of variability (CV): Intra-assay CV and Inter-assay CV. The measurements of 3 known concentrations of recombinant NGAL (250, 500 and 1000 ng/ml) were repeated 20 times on one slide to determine the intra-assay precision. Twenty replicates of each sample from five separate slides were used to calculate the inter-assay CV.

The intra-assay is used to determine the spot-to-spot variability, while the inter-assay shows the slide-to-slide reproducibility. As shown in Table 1, the intra-assay CV was ranged from 1.83% to 4.54% with the average intra-assay CV of 2.67%. The inter-assay CV of this system was ranged from 1.06% to 1.67% and the average was 1.28%. Typically, for assay quality control, the intra-assay CV of less than 10% is generally acceptable, while the inter-assay CV should be less than 15% (Bonham et al, 2009; Murray et al, 1993). Thus, our intra-, and the inter-assay CVs indicate spot-to-spot and slide-to-slide consistency. Furthermore, the error of the measurement was less than 10%. As a result, the quantification of NGAL protein using our slide-based immunoassay was considered reasonably accurate.

3.3 *Comparison of the slide-based immunoassay with ELISA*

Table 2 presents a comparison between our system and traditional ELISA. The total assay time of our slide-based system is 2 hours less than that of ELISA. The high surface area to volume ratio of this miniaturized system resulted in shorter diffusional distances, which allowed the antibody and the protein to conjugate more rapidly and, hence, decreased the analysis time (Bange et al, 2005). The assay time of the slide-based system could be further reduced to approximately 1 hour (30 minutes of sample incubation and a 30 minute incubation of a mixture of the secondary antibody conjugated with biotin and fluorescent-

labeled streptadvidin) (See Supplementary B). At this lower assay time, the linear range and the slope of the calibration curve were similar to those of the standard assay time, albeit 35% reduction of the fluorescence intensity. Thus, if a rapid screening is desired, the sensitivity of the system may need to be sacrificed.

Since the reagent consumption of this system is 200 times less than that of the ELISA method, the cost of expensive antibodies as well as the operating cost can be drastically reduced. Another advantage of the slide-based system is that the washing step can be done easily and quickly. The remaining antibody, protein and wash solutions can be completely removed, preventing any possible cross-contamination between spots, which sometimes occurs in the traditional well-based assay.

The measurement interval of ELISA was found to be much lower than the actual NGAL concentrations observed in human serum and urine (Dent et al, 2007; Bennett et al, 2008). As a result, a dilution step is required prior to an analysis using ELISA, leading to a cumbersome preparation step and possible measurement errors. On the other hand, no dilution is necessary when using the slide-based system to detect plasma NGAL because its measurement interval well covers the range of NGAL concentrations found in normal human and AKI patients.

3.4 *Analysis of NGAL in plasma and urine samples*

The measurement of NGAL in 40 plasma and urine samples was carried out using the slide-based system and validated against the measurement of the same samples using ELISA. For the slide-based system, one slide could analyze up to 22 samples in triplicate in parallel with 7 standard protein dilution series (0 – 1000 ng/ml), in which the measurement was repeated twice. The plasma samples were not diluted, while the urine samples had to be diluted 50 fold in PBS before the analysis. As expected, the levels of both plasma and urine NGAL of the patients with AKI were significantly higher than those of the healthy controls

(Table 2). This finding is consistent with previous studies (Devaraja, 2010). The average plasma NGAL concentrations in healthy controls and AKI patients were comparable between the two methods. In addition, the concentrations of plasma NGAL measured using the slide-based system matched well with the values from ELISA, as indicated by the slope of nearly 1 and the correlation coefficient (r^2) of 0.99 (Figure 3). This result demonstrates good agreement between the two methods in the measurement of plasma NGAL, thus confirming the good performance of this assay system.

In contrast, significantly higher urine NGAL concentrations were observed with the slide-based system, as compared to those of ELISA ($r^2 = 0.89$). The discrepancy between the two methods may have come from the interference caused by a matrix effect. The previous study has demonstrated that antibody binding and assay performance could be affected by the variability of urine matrix components such as organic compounds, pH, and electrolytes (Sviridov et al, 2009). To overcome this problem, sample dilution was suggested. Interestingly, the accuracy of the measurement was improved at the higher levels of dilution (Taylor et al, 2012). In this study, due to the differences in the measurement intervals (Table 2), the urine samples were diluted 800 fold for ELISA, while only 50 fold dilution was required for the slide-based assay. As a result, the presence of more concentrated urinary components might have contributed to the interference of the immunoassay performed on the slide-based system, leading to the over-prediction of urine NGAL concentration. For a better correlation of urine NGAL measurements between the two systems, more dilution of the urine samples is necessary for the slide-based system. Unfortunately, the same level of dilution is not possible because the concentration of urine NGAL in the diluted samples would be below the limit of detection under the slide-based system. Unlike urine samples, the variability of matrix components in plasma and serum is negligible (Hortin et al, 2005). Even

though a dilution step was only applied to the plasma samples for ELISA, the measured plasma NGAL concentrations were similar between the two methods.

Another possible explanation is that a compound or protein structurally similar to the analyte might cross-react with the capture antibody used in the slide-based system, leading to false positive (Dodig, 2009). Since the sources of the capture antibodies of the slide-based system and ELISA were different, it is possible that the binding between NGAL and antibody might have occurred at different epitopes. As a result, positive interference only occurred in our system, but not in ELISA. However, these are only hypotheses. Further studies are needed to identify the true cause of the urine interference.

In conclusion, the immunoassays performed on the slide-based platform gave satisfactory results, especially for the quantification of plasma NGAL. When compared with ELISA, the new assay system requires much smaller quantities of antibody and less processing time. Unfortunately, like most analytical techniques, the slide-based system has some limitations due to possible variability in the capture antibody concentrations from manual spotting of antibody. Nano-liter liquid dispensing equipment would certainly overcome this problem. Nonetheless, we believe that our slide-based immunoassay system has the potential to be used as a more rapid and inexpensive alternative to measure plasma NGAL for early diagnosis of AKI. This platform could be a bed-side tool for an intervention trial of AKI in the future.

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DISCLOSURE

The authors do not have any financial relationship with any companies that may have financial interest in the information provided by this manuscript.

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Table 1 Intra- and Inter-assay CVs and % errors of the slide-based platform

	Intra-assay			Inter -assay		
Sample	1	2	3	1	2	3
Actual conc.	250	500	1000	250	500	1000
n	20	20	20	20	20	20
Mean (ng/ml)	264.24	508.10	950.99	263.30	508.16	952.56
% CV	4.54	1.83	1.64	1.11	1.67	1.06
% error	5.69	1.62	4.90	5.32	1.63	4.74

Abbreviation: CV, coefficient of variability.

Table 2 Comparison between ELISA and the slide-based immunoassay for NGAL measurement

	ELISA*	**SLIDE**
Time	4½ hours	2½ hours
Volume of reagents	100 µl/sample	0.5 µl/sample
Linear Range (ng/ml)	0.156 – 10	50 – 1000
Correlation coefficient (R^2)	0.9922	0.9903

*: based on the manufacturer's instruction

Table 3 Plasma and urine NGAL (ng/ml) obtained by ELISA and the slide-based system

	Healthy control	AKI
N	20	20
Age	29.05 (23-40)	68.85±19.04
Female	80%	45%
APCHE II score	-	17.30 ±7.65
Sepsis (n [%])	-	13 [65]
Shock (n [%])	-	8 [40]
RRT (n [%])	-	5 [20]
pNGAL (ng/ml)	57.39±10.99 (ELISA) 51.20±10.07 (Slide)	356.37±154.24 (ELISA) 353.14±153.01 (Slide)
uNGAL (ng/ml)	23.16±8.16 (ELISA) 43.58 ± 21.30 (Slide)	1071.59±1006.24 (ELISA) 1500.83 ± 902.65 (Slide)

Abbreviations: pNGAL, Plasma NGAL; uNGAL , urine NGAL.
APACHE II, Acute Physiology and Chronic Health Evaluation II
RRT, Renal Replacement Therapy

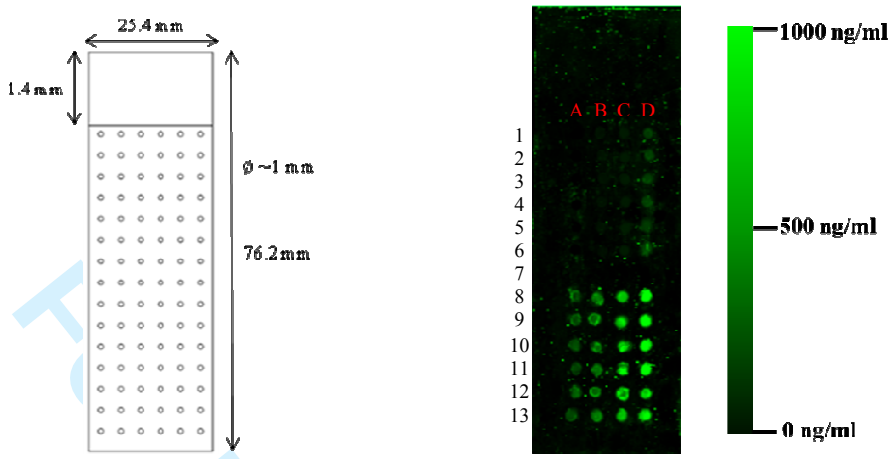


Figure 1: A. Schematic diagram of the antibody immobilized slide. B. Fluorescence image of the slide used to generate a standard curve: A1 - A6 were regarded as "negative", while the spots on row 7 were used for "blank". The rest were used to measure various concentrations of NGAL in the samples, specifically, B1 - B6 for 10 ng/ml, C1 - C6 for 25 ng/ml, D1 - D6 for 50 ng/ml, A8 - A13 for 100 ng/ml, B8 - B13 for 200 ng/ml, C8 - C13 for 500 ng/m, and D8 - D13 for 1000 ng/ml.

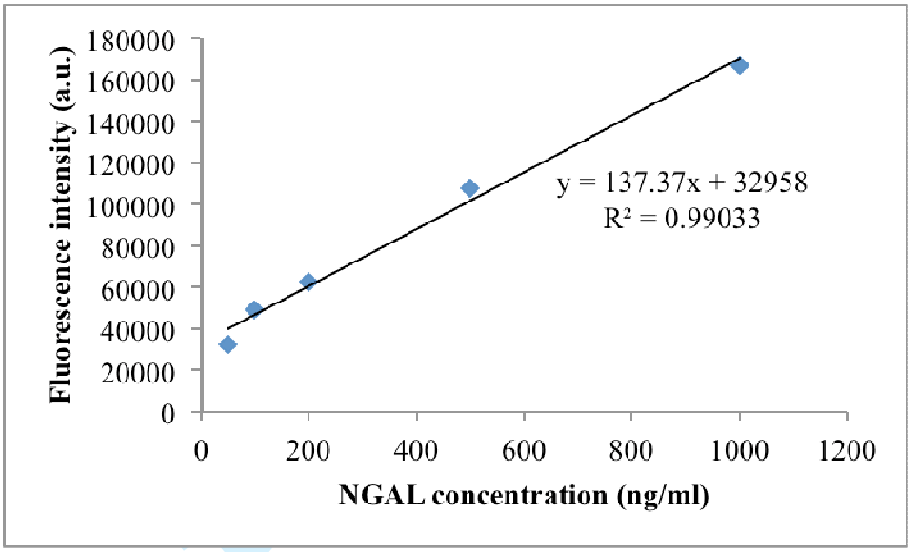


Figure 2: Calibration curve of NGAL using the slide-based immunoassay platform

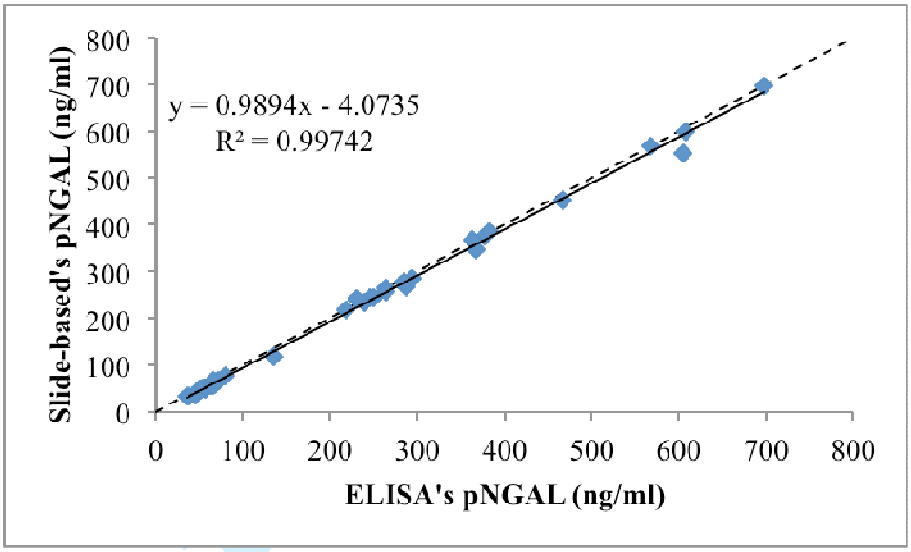


Figure 3: Scatter plot of plasma NGAL concentrations measured using ELISA and the slide-based platform

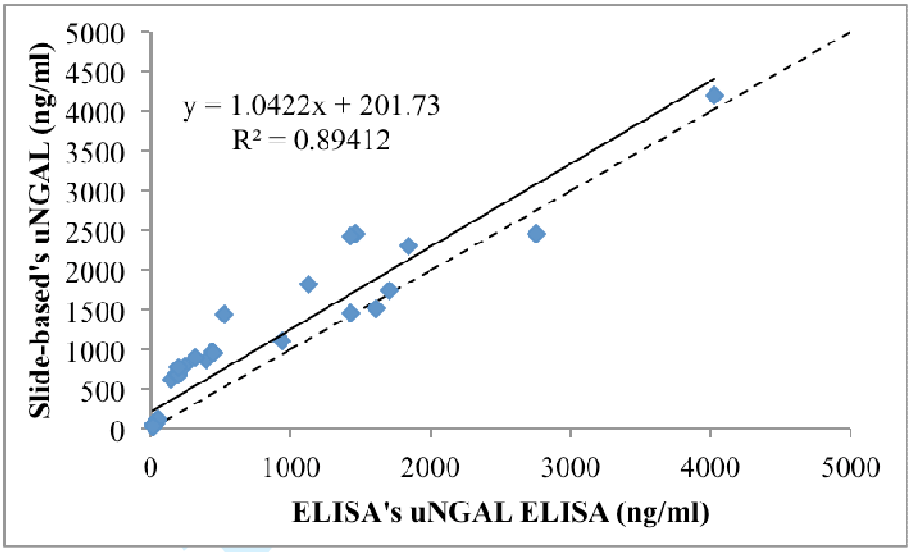


Figure 4: Scatter plot of urine NGAL concentrations measured using ELISA and the slide-based platform

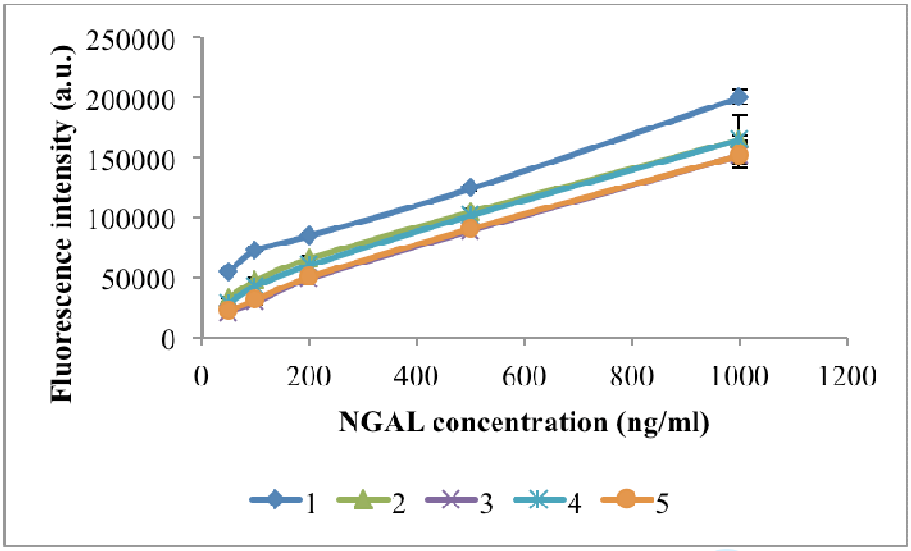
Supplementary Information

	A	B	C	D
1	neg.	10	25	50
2	neg.	10	25	50
3	neg.	10	25	50
4	neg.	10	25	50
5	neg.	10	25	50
6	neg.	10	25	50
7	blank	blank	blank	blank
8	100	200	500	1000
9	100	200	500	1000
10	100	200	500	1000
11	100	200	500	1000
12	100	200	500	1000
13	100	200	500	1000

Supplementary A: Experimental design of the slide-based immunoassay to construct a calibration curve. "neg." refers to negative control or 0 ng/ml of NGAL. "blank" refers to the spots with no capture antibody. The numbers, from "10 – 1,000" refer to the concentration of recombinant NGAL used in this study.

Supplementary B1: Different sets of operating conditions. Note that condition 1 is the standard condition for the slide-based immunoassay platform.

	Sample	Biotinylated antibody	Labeled streptavidin	Biotinylated antibody mixed with Labeled streptavidin	Total time
1	1 hour	1 hour	½ hour	-	2½ hours
2	1 hour	-	-	1 hour	2 hours
3	1 hour	-	-	½ hour	1½ hours
4	½ hour	-	-	1 hour	1½ hours
5	½ hour	-	-	½ hour	1 hour



Supplementary B2: Calibration curves of NGAL acquired under different sets of operating conditions.