Development of a rapid sandwich Enzyme Linked Immunoassay procedure for the highly sensitive detection of human Lipocalin-2/NGAL

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Abstract

A rapid sandwich enzyme linked immunoassay (ELIA) procedure was developed for the highly sensitive detection of human Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL). It reduced the total immunoassay duration by more than 3-fold and detected LCN2 in the pathophysiological range of 2.5-5120 pg/mL with an analytical sensitivity of 7 pg/mL, which is about 11-fold better than that of the commercially-available conventional enzyme linked immunosorbent assay (ELISA). The enhanced analytical performance of developed ELIA procedure is due to the leach-proof covalent crosslinking of anti-human LCN2 antibody to the 3-aminopropyltriethoxysilane (APTES)-functionalized microtiter plate (MTP) by 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride. The multisubstrate-compatible ELIA procedure enables LCN2 assay on different types of polymeric substrates (inert, hydrophilic and hydrophobic) using the modified MTP format. The developed ELIA procedure can be similarly employed to develop rapid and highly sensitive immunoassays for other disease biomarkers and analytes in clinical and industrial settings.

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1. Introduction

LCN2/NGAL is a small 25 kDa secretory glycoprotein found in neutrophils and certain epithelia such as renal tubules [1]. LCN2 expression is dramatically increased in ischemic [2] or nephrotoxic injury [3]. It is an early and sensitive biomarker for acute renal injury [4,5] as its levels rise in urine and blood just within a few hours. Higher levels of LCN2 may lead to acute renal failure. It is also relevant in cases of cardiovascular surgery; coronary heart disease [6]; kidney transplants [7,8]; bacterial infections [9]; fatty liver disease; obesity and its associated pathologies; several types of cancers such as breast [10], pancreatic [11], ovarian [12] and oesophageal [13]; and, obesity-induced metabolic disorders [14] such as insulin resistance, type 2 diabetes mellitus and cardiovascular disorders. The prognosis of acute renal failure is usually done by creatinine or cystatin C measurements but they respond only after a few days of the renal function deterioration. The down-regulation of LCN2 is also a diagnostic marker for many diseases such as glioblastoma [15], where LCN2 is down-regulated as a subsidiary mechanism of enhancing chemoresistance in glioblastoma cells. The LCN2 threshold is also down-regulated in ovarian cancer [12] and microglial cells [16]. Studies have also suggested that LCN2 is a predictive marker for excessive exposure to high electromagnetic fields that significantly decreases the body threshold of LCN2 [17]. Therefore, LCN2 has an important prognostic and diagnostic value as a biomarker. The LCN2 concentrations in urine and plasma of healthy persons are in the ranges of 0.7-9.6 ng/mL and 37–106 ng/mL, respectively. It rises immediately after renal injury in urine and plasma, where its concentrations are in the range of 110-40,000 ng/mL and 25-3491 ng/mL, respectively. However, in case of acute renal failure, the NGAL concentrations in urine and plasma are above 350 ng/mL and above 400 ng/mL, respectively.

Several sandwich ELISA kits have been made in the last few years, which can reliably determine the pathophysiological LCN2 levels in the biological samples. However, we have developed a rapid and highly-sensitive sandwich ELIA procedure based on the covalent binding of anti-human LCN2 antibodies on the APTES-functionalized surface (Fig. 1). It has 11-fold better sensitivity, 3-fold reduced overall assay duration, greater reproducibility and lesser variability than the conventional ELISA that is being used in several commercially existing kits. The commercially available human LCN2 sandwich ELISA kit, containing all the assay components, was employed. The developed and conventional procedures were evaluated under the same conditions at the same time using the same assay components. The multi-substrate compatibility of the developed ELIA enabled LCN2 assays on various types of commercially-relevant substrates using the modified microtiter plate (MTP) [18]. The developed ELIA procedure will be of great utility to develop rapid and highly sensitive immunoassays for other disease biomarkers and analytes.

2. Materials and Methods

2.1 Materials

EDC, N-hydroxysuccinimide (NHS), N-hydroxysulfosuccinimide (sulfoNHS), phosphate buffered saline (PBS, 0.1M, pH 7.4), 2-(N-morpholino)ethanesulfonic acid (MES, pH 4.7), 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit and bovine-serum albumin (BSA) were purchased from Thermo Scientific. Potassium hydroxide (KOH), 3-APTES, Tween 20 and absolute ethanol were obtained from Sigma-Aldrich. The human LCN2/NGAL Duoset kit, with all sandwich ELISA components, was procured from RnD Systems, USA. Anti-human LCN2 antibody was used as capture antibody, whereas biotinylated anti-human LCN2 detection antibody and streptavidin-conjugated horseradish peroxidasewere employed for detection. 0.1M PBS, pH 7.4 with 1% (v/v) BSA was used for
reconstituting all assay components, whereas ultrapure water (UPW) (18 MΩ, Direct Q, Millipore) was used for preparing buffers, KOH and 3-APTES. EDC, NHS and SNHS were reconstituted in 0.1M MES buffer, pH 4.7.

Poly(methyl methacrylate) (PMMA), polystyrene (PS) and zeonexTM (Znx) slides were purchased from Microfluidic Chip Shop GmbH, Germany. Polycarbonate (PC) and cellulose acetate (CA) were received from VTT, Finland, whereas Zeonor (Znr) was procured from Zeon Chemicals, Germany. Pressure-sensitive adhesive (PSA) was obtained from Adhesive Research. Bottomless 96-well MTPs were purchased from Greiner Labortechnik, Germany. The assay temperature was maintained at 37°C using a thermostat from Labnet International Inc., USA. Absorbance was measured using Tecan Infinite M200 Pro instrument procured from Tecan GmbH, Austria.

![Image](image.png)

Fig. 1. Schematic of covalent crosslinking of antibody in the developed human Lipocalin-2 sandwich ELIA.

2.2 Methods

2.2.1 Preparation of anti-human LCN2 antibody-bound MTP

Each well of the 96-well MTP was treated with 100 μL of absolute ethanol for 5 min at 37°C and washed five times with 300 μL of UPW. Subsequently, each well was treated with 100 μL of 1.0% (w/v) KOH at 37°C for 10 min followed by five washings with 300 μL of UPW. The KOH-treated wells were then functionalized with amino groups by incubating with 100 μL of 2% (v/v) APTES per well at room temperature for 1 h inside the fume cabinet. The amine-functionalized plate was subsequently washed five times with 300 μL of DIW in order to remove excess unbound 3-APTES from the surface. Thereafter, anti-human LCN2 (990 μL of 2 μg/mL) was incubated with 10 μL of EDC (4 mg/mL) for 15 min at 37°C.
The resulting EDC-activated anti-human LCN2 antibody solution was added to each of the functionalized wells (100 μL) and incubated for 1 h at 37°C. The anti-human LCN2 antibody-bound wells were then washed six times with 400 μL of PBST (0.05% Tween 20 in PBS, pH 7.2).

2.2.2 Sandwich ELIA procedure

The anti-human LCN2 antibody-bound MTP wells were blocked with 1% (v/v) BSA, diluted in 0.1M PBS, pH 7.4, for 30 min at 37°C and subsequently washed six times with 400 μL of PBST. 100 μL of each of the varying concentrations of human LCN2 (2.5-5120 pg/mL), prepared in 0.1M PBS with 1% BSA, pH 7.4, were incubated in the anti-human LCN2 antibody-coated MTPs for 1 h at 37°C followed by washing with 400 μL of PBST six times. 100 μL of biotinylated anti-human LCN2 detection antibody (100 ng/mL) was then added and incubated for 1 h at 37°C followed by six washes with 400 μL of PBST. 100 μL of SA-HRP, diluted 1:200, was then added to each well and incubated for 20 min at 37°C followed by six washes with 400 μL of PBST. The TMB substrate was then added (as per the manufacturer’s guidelines) and the enzyme-substrate reaction was stopped after 20 min by adding 50 μL of 2N H2SO4. The absorbance was measured at 450 nm with reference at 540 nm (to eliminate the optical imperfections at well-to-well level). All the experiments were done in triplicate with zero ng/mL human LCN2 (in 0.1M PBS, pH 7.4 with 1% BSA) as control, whose absorbance was subtracted from all the assay values.

The preparation of modified MTPs, used to perform sandwich human LCN2 ELIA on different polymeric substrates, has been described elsewhere [18]. The conventional sandwich ELISA procedure was followed as per the manufacturer’s guidelines provided in the product information sheet without any modification.

2.2.3 Assay performance analysis

All datasets obtained from the developed ELIA and conventional ELISA procedures were subjected to four-parameter logistic function-based standard curve analysis in the Sigma Plot software, version 11.2. The EC50, Rsqr, hillslope values were determined from the report generated by the software. The analytical sensitivity was calculated by [mean absorbance of blank + 3(standard deviation of the blank)]. The variability was reported as percentage coefficient of variation (%CV). The intra-assay and inter-assay variability were calculated from six assay repeats (in triplicate) on a single day and on six different days, respectively. All the assays were carried out in triplicate.

3. Results and Discussion

The developed sandwich ELIA detected human LCN2 in the range of 2.5-5120 pg/mL (r²=0.99) with an analytical sensitivity of 7 pg/mL [Fig. 2(a)]. The intra-assay and inter-assay variability were 1.1-11.8 and 1.9-14.5, respectively, while the percentage recovery for the various concentrations of human LCN2 was between 85 and 110. The maximal half-effective concentration (EC50) obtained from the assay curve was very low i.e. 624 pg/mL, which demonstrated the high sensitivity of developed ELIA. The high sensitivity was mainly due to the covalent crosslinking of capture antibody that led to the leach-proof binding of antibodies to the substrate with high immobilization density. The developed ELIA procedure is an improvement of our previous immunoassay procedure [19,20], which requires additional steps of oxygen plasma treatment and silanization at 80 °C under vacuum. The developed ELIA have no such limitations and can be employed in any bioanalytical lab. The optimization of antibody-crosslinking on APTES-functionalized MTP further revealed that EDC alone crosslinks antibodies more efficiently than
EDC in combination with sulfoNHS or NHS [Fig. 2 (b)]. 2% APTES was the optimized APTES concentration [Fig. 2 (c)], while 1 h was the optimum silanization time [Fig. 2 (d)].

Fig. 2. (a) Comparison of the developed sandwich ELIA for human LCN2 with the conventional ELISA. (b) Comparison of various EDC-based antibody crosslinking strategies for the developed human LCN2 sandwich ELIA. (c) Optimization of APTES concentration used for silanization. (d) Optimization of duration of silanization. (e) Developed human LCN2 sandwich ELIA on
different types of commercially-relevant substrates i.e. hydrophobic (PS, PC, PMMA), hydrophilic (CA), and inert (cyclo-olefin polymers; with trade names of Zeonex and Zeonor). All the experiments were done in triplicate. The error bars represent standard deviations.

The performance of the developed ELIA was compared to that of conventional ELISA (Table 1) by performing both the immunoassays simultaneously on the same day under the same conditions. The dynamic range of conventional human LCN2 ELISA was 40-5120 pg/mL ($r^2=0.99$), while its analytical sensitivity was 80 pg/mL that was about 11-fold less sensitive than that of developed ELIA. Moreover, the overall immunoassay duration of developed ELIA (~6 h) was greater than 3-fold lesser than that of conventional ELISA (~20 h). Additionally, the developed ELIA had less variability than that of conventional ELISA.

In industrial settings, the passively adsorbed antibody-coated MTPs are kept refrigerated for about 2 weeks so that they can be used immediately to detect specific analytes whenever required. However, the developed ELIA has completely eliminated the storage of antibody-coated MTPs as the overall immunoassay, including the antibody immobilization, takes only 6 h. It enhances the quality of immunoassays by eliminating the storage-based effects. The developed human LCN2 ELIA was performed on various types of polymeric substrates i.e. hydrophobic (PS, PC, PMMA), hydrophilic (CA) and inert (cyclo-olefin polymers with trade names of Zeonex and Zeonor) using the modified MTP format [18] [Fig. 2 (e)]. However, as these substrates have varying thicknesses, the human LCN2 assays performed on them cannot be compared because the absorbance is dependent on the optical path length.

The developed human LCN2 ELIA is the most sensitive assay reported so far based on its comparison with various commercially-available ELISA kits (Table 2). However, the commercial kits may have different assay components such as monoclonal or polyclonal antibody types, which may be responsible for their lower sensitivity as the nature of the antibody determines the antigen capture efficiency and thus the sensitivity and specificity of an immunoassay. The developed ELIA procedure will be of tremendous utility to develop rapid and highly sensitive immunoassays for other disease biomarkers and industrial analytes.

4. Conclusion

A rapid sandwich ELIA procedure, based on the covalent crosslinking of anti-human LCN2 antibodies to APTES-functionalized microtiter plate, was developed for the highly sensitive detection of human LCN2. It is the most sensitive assay format reported so far for human LCN2. It was about 11-fold more sensitive, 3-fold more rapid and more reproducible than the conventional ELISA procedure, which is being used in most commercially-available diagnostic kits. It has completely eliminated the requirement of storing antibody-bound MTPs in industrial settings. It can be further employed to perform human LCN2 assay on various types of commercially-relevant polymeric substrates using the modified MTP format. The developed ELIA has much better analytical performance than the conventional ELISA and can be effectively used for immunoassays in clinical and industrial settings.

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Table 1. Analytical comparison of the developed sandwich ELIA for human LCN2/NGAL with the conventional ELISA.

|                          | Developed ELIA | Conventional ELISA |
|-------------------------|----------------|--------------------|
| Detection Range (pg/mL) | 2.5-5120       | 40-5120            |
| LOD (pg/mL)             | 3              | 44                 |
| Analytical Sensitivity (pg/mL) | 7       | 80                 |
| EC50 (pg/mL)            | 624            | 821                |
| % CV                    |                |                    |
| Intra-day (n=6)         | 1.1-11.8       | 1.6-17.2           |
| Inter-day (n=6)         | 1.9-14.5       | 2.4-18.1           |
| Assay duration (hours)  | ~6             | ~20                |
| Assays on various substrates | Yes     | No                 |

Table 2. A comparative analysis of the developed human LCN2/NGAL sandwich ELIA with various commercially-available sandwich ELISA kits.

| Manufacturer                        | Antibody immobilization technique | LOD*     | Refer to                      |
|-------------------------------------|-----------------------------------|----------|-------------------------------|
| Covalent assay                      | Chemical crosslinking              | 2.5 pg/mL| Reported in this manuscript   |
| Passive assay                       | Passive adsorption                 | 40 pg/mL | Reported in this manuscript   |
| R&D Systems, Inc                    | Passive adsorption                 | 78 pg/mL | www.rndsystems.com            |
| Boster Biological Technology Co., Ltd. | Passive adsorption             | 10 pg/mL | www.immunoleader.com          |
| Antibody and Immunoassay Services   | Passive adsorption                 | 0.4 ng/mL| www.antibody.hku.hk           |
| Meso Scale Diagnostics              | Passive adsorption                 |          | www.mesoscale.com             |
| BioPorto Diagnostics                | Passive adsorption                 | 0.2 ng/mL| www.biopporto.com             |
| CycLex Co., Ltd                     | Passive adsorption                 | 26.7 pg/mL| www.cyclex.co.jp              |
| BioVendor                           | Passive adsorption                 | 20 pg/mL | www.biovendor.com             |
| Argutus Medical                     | Passive adsorption                 | 0.4 ng/mL| www.argutusmed.com            |

*the lowest concentration detected by the assay.
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