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Serological Measurement of Poly-IgA Immune Complex Levels in IgA Nephropathy and IgA Vasculitis

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Abstract

Both IgA nephropathy and IgA vasculitis, formerly known as Henoch-Schönlein purpura, are immune deposition diseases. IgA nephropathy is caused by the deposition of aberrantly formed poly-IgA complexes from blood circulation to the kidney glomerulus; IgA vasculitis is characterized by IgA-dominant immune deposits to small vessels of the skin and other organs, including the kidney. Therefore, measuring the disease-causing poly-IgA contents in the plasma is needed to study these conditions. However, while clinical tests for the level of total plasma IgA are routinely performed, methods for specific detection of poly-IgA contents are unavailable in clinical medicine. In this protocol, we describe a practical solution for measuring poly-IgA in patient samples. The new method is based on the biological selectivity of IgA Fcα receptor I (FcαRI/CD89) toward poly-IgA species, in contrast to its relatively low affinity for normal monomeric IgA. By devising recombinant CD89 ectodomain as the "capturing" probe, we validated the feasibility of the assay for measuring plasma poly-IgA levels in a 96-well format. The methodology was able to differentiate plasma samples of IgA nephropathy, or related IgA vasculitis, from those of other autoimmune kidney disease types or from healthy controls. Moreover, the measured poly-IgA indices not only correlated with the severity of IgA nephropathy, but the levels also trended lower following corticosteroid or immunosuppressant treatments of patients. Therefore, we anticipate the new assay will provide useful measurements of the IgA nephropathy disease activity index for stratifying disease severity or for evaluating treatment response.

Keywords: Poly-IgA immune complex, CD89/IgA Fc receptor, IgA nephropathy, IgA vasculitis/Henoch-Schönlein purpura, Circulating IgA immune activity index, ELISA method

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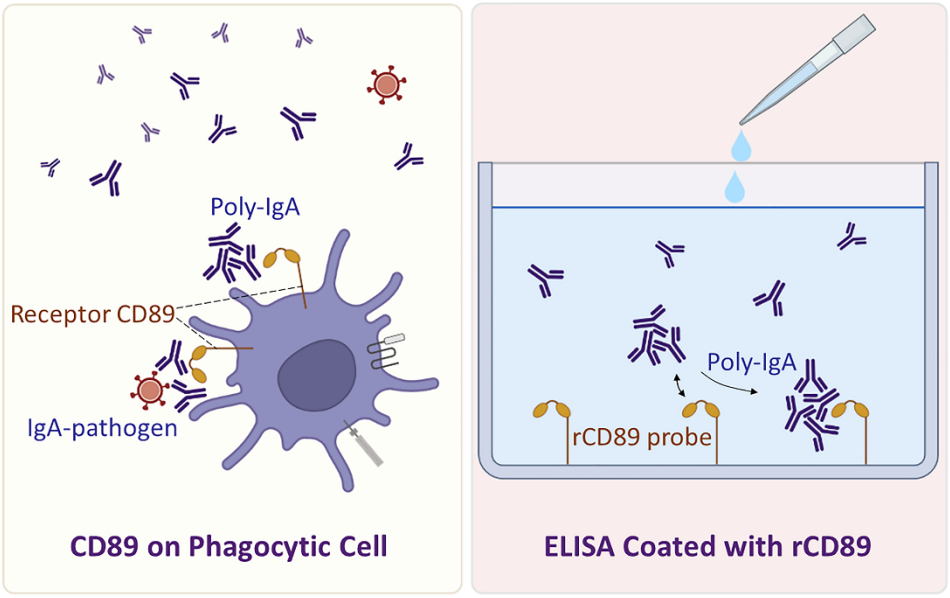
Graphical abstract:



Background

IgA nephropathy is the most common form of primary glomerulonephritis worldwide (Lai *et al.*, 2016). The diagnosis requires renal biopsy and there are no validated diagnostic serum or urine biomarkers for IgA nephropathy. Among recognized risk factors, high levels of galactose-deficient IgA1 (Gd-IgA1) and excessive formation of poly-IgA immune complexes in circulation are associated with kidney glomerular deposition (Magistroni *et al.*, 2015). The observations of recurrent IgA deposition in kidney grafts in transplant recipients with IgA nephropathy strongly suggest the extrarenal origin of IgA as the source of the renal deposits (Moroni *et al.*, 2019). This notion is further supported by evidence of circulating immune complexes in patients sharing the same immunoglobulin classes with IgA extracted from the kidney (Kanatsu *et al.*, 1983). Local immune reactivities to the IgA deposits in the glomerulus stimulate proliferation of mesangial cells, synthesis of extracellular matrix, and infiltration of inflammatory cells (Magistroni *et al.*, 2015).

Meanwhile, circulating IgA immune complexes are catabolized primarily by the mononuclear phagocyte system via IgA Fcα receptor I (FcαRI/CD89) (Chen *et al.*, 2018). CD89 is a type I transmembrane glycoprotein broadly expressed on the surface of myeloid cells, including monocytes/macrophages, dendritic cells, Kupffer cells, neutrophils, and eosinophils (Maliszewski *et al.*, 1990; Monteiro and Van De Winkel, 2003; Bakema and van Egmond, 2011). This IgA-specific receptor binds both IgA1 and IgA2 through its N-terminus Ig domain, which interacts with the Cα2/Cα3 junction of IgA (Herr *et al.*, 2003). Previously, we and others demonstrated that CD89 exhibits higher affinities to polymeric IgA than to monomeric IgA, allowing phagocytes to selectively capture poly-IgA complexes (Reterink *et al.*, 1997; Zhang *et al.*, 2021).

To this end, we constructed a recombinant (r) CD89-based affinity probe for serological detection of poly-IgA immune complexes in clinical samples, namely from patients with IgA nephropathy and IgA vasculitis, formerly known as Henoch-Schönlein purpura (HSP) (Zhang *et al.*, 2020). In our study, we demonstrated that rCD89 can distinctively capture poly-IgA in the plasma samples, making this rCD89 probe suitable for measuring only the disease-causing poly-IgA contents without the interference of background signals from normal IgA species. The probe was mounted on ELISA plates and the tests were performed directly with diluted plasma samples. Using this high-throughput test, we demonstrated significantly elevated levels of poly-IgA complexes in IgA nephropathy and HSP samples, as compared to either healthy controls or samples from other kidney disease types (Figure 1). The results outperformed those obtained from measurements of either total IgA or Gd-IgA1 levels regarding disease

correlation. It is important to note that despite the recognition of circulating poly-IgA complexes being prone to deposition in the kidney and elsewhere, clinical tests for their levels are unavailable. Research labs have been using size-exclusion chromatography (SEC) to isolate and characterize high-molecular weight IgA complexes. However, the procedures are cumbersome and the quality of poly-IgA separation from their monomeric and dimeric counterparts by SEC is poor (Reterink *et al.*, 1997; Novak *et al.*, 2005, 2011). In addition, chromatography requires pre-extraction of IgA and these additional steps have the tendency to introduce experimental artifacts of IgA self-aggregation (Hui *et al.*, 2015). In contrast, our recombinant CD89-directed ELISA is a robust assay that can be easily automated and further adapted to clinical applications. Here we describe the experimental workflow in a research lab setting.

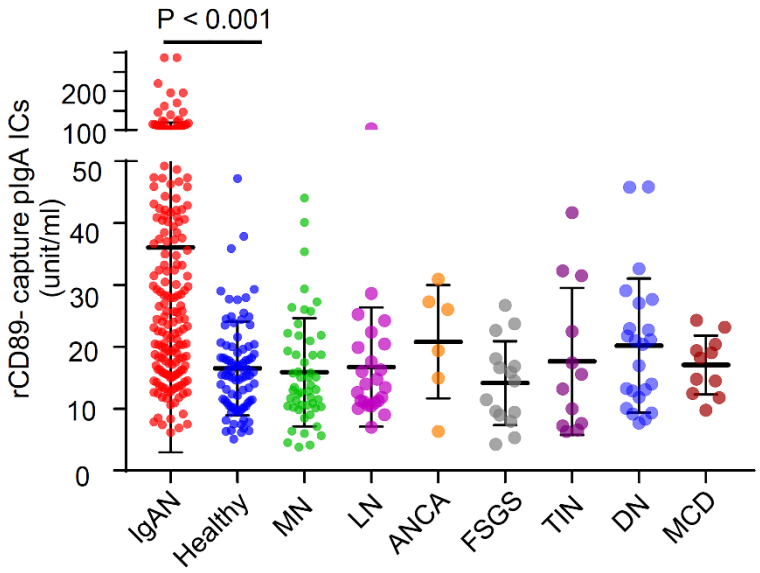


Figure 1. Comparison of plasma poly-IgA levels among different kidney diseases.
The rCD89-directed ELISA kit detects higher poly-IgA levels in plasma samples of IgAN patients, as compared to samples of healthy controls or non-IgAN kidney disease types, including membranous nephropathy (MN), lupus nephritis (LN), ANCA nephritis, Focal Segmental glomerulosclerosis (FSGS), tubulointerstitial nephritis (TIN), diabetic nephropathy (DN), and minimum change disease (MCD). (Note that the figure was adapted from Zhang *et al.*, 2021)

Materials and Reagents

1. Disposable tips
   a. 10 µL capacity (Thermo Fisher Scientific, QSP, catalog number: 104-Q, Rockford, US)
   b. 200 µL capacity (Thermo Fisher Scientific, QSP, catalog number: TF140-200-Q, Rockford, US)
   c. 1 mL capacity (Thermo Fisher Scientific, QSP, catalog number: 112NXL-Q, Rockford, US)
2. 1.5 mL Microcentrifuge tube (Thermo Fisher Scientific, Invitrogen, catalog number: AM12400, Carlsbad, US)
3. 100 mm culture dish (Corning, Falcon, catalog number: 353003, Glendale, US)
4. Amicon Ultra-15 centrifugal filter units, 10 kDa MWCO (Sigma, Millipore, catalog number: UFC901024, Darmstadt, Germany)
5. Carbonate bi-carbonate coating buffer (Medicago, catalog number: 09-8922-100, Uppsala, Sweden)
6. Bovine serum albumin (Sigma, catalog number: 90604-29-8, Saint Louis, US)
7. Tween-20 (Thermo Fisher Scientific, catalog number: BP337500, Carlsbad, US)
8. pcDNA3 expression vector (Thermo Fisher Scientific, Invitrogen™, catalog number: V79020, Carlsbad, US)
9. HEK293 cells (ATCC, catalog number: CRL-1573, Manassas, US)

10. Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Invitrogen, catalog number: 11668027, Carlsbad, US)
11. Geneticin/G418 (Thermo Fisher Scientific, Gibco, catalog number: 11811023, Carlsbad, US)
12. Dulbecco's Modified Eagle's Medium (Life Sciences, Corning, catalog number: 10-017-CV, Glendale, US)
13. Fetal bovine serum (Thermo Fisher Scientific, Gibco, catalog number: 10099141, Carlsbad, US)
14. Commercial CD89 protein, human, recombinant (Sino Biological, catalog number: 10414-H08H, Beijing, China)
15. Human IgA1 standard, myeloma (Sigma, catalog number: 400109, Saint Louis, US)
16. PBS powder (Sigma, catalog number: P3813, Saint Louis, US)
17. 3×FLAG peptide (Sigma, catalog number: F4799, Saint Louis, US)
18. ANTI-FLAG M2 affinity gel (Sigma, catalog number: A2220, Saint Louis, US)
19. Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, catalog number: 66380, Carlsbad, US)
20. Bio-Rad Protein Assay Dye Reagent (Bio-Rad, catalog number: #5000006, Hercules, US)
21. Primary antibodies and secondary antibodies
    a. HRP-conjugated anti-DDDDK tag antibody [M2] (Abcam, catalog number: ab49763, Waltham, US)
    b. HRP-conjugated anti-human IgA antibody [1H9] (Abcam, catalog number: ab7383, Waltham, US)
    c. Recombinant anti-CD89 antibody (Abcam, catalog number: ab124717, Waltham, US)
22. ECL Western Blotting Substrate (Thermo Scientific, catalog number: 32209, Carlsbad, US)
23. Nunc MaxiSorp 96-Well plates (Thermo Fisher Scientific, catalog number: 44-2404-21, Glendale, US)
24. Immuno Clear Standard Modules (Thermo Scientific, catalog number: 468667, Carlsbad, US)
25. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate set (BD, catalog number: 555214, Franklin Lakes, US)
26. Sulfuric acid (Thermo Scientific, catalog number: N600, Carlsbad, US)
27. Penicillin and streptomycin (Thermo Scientific, catalog number: 15140122, Carlsbad, US)
28. Cell culture mediums (see Recipes)
    Growth medium
    Selection medium
    Cryopreservation medium
29. 0.05 M carbonate sodium buffer (pH 9.6) (see Recipes)
30. 0.01 M Phosphate Buffered Saline (PBS) buffer (see Recipes)
31. ELISA washing buffer (0.1% PBST buffer, pH 7.4) (see Recipes)
32. ELISA blocking buffer (1% BSA/PBST buffer) (see Recipes)
33. ELISA stop solution (1 M H_2SO_4) (see Recipes)

Equipment

1. -80°C freezer (*e.g.*, Thermo Scientific Forma 900 Series)
2. Fixed volume single-channel pipettes (*e.g.*, Thermo Fisher Scientific, catalog number: 4651140)
3. Refrigerated tabletop centrifuge (*e.g.*, Thermo Sorvall Legend XTR refrigerated centrifuge)
4. Microplate absorbance reader (*e.g.*,Bio-Rad, catalog number: 1681130XTU)
5. CO_2 incubators (*e.g.*, Thermo Fisher Scientific, catalog number: 51032875)
6. Liquid nitrogen tank (*e.g.*, Thermo Scientific)
7. Gel imaging system (*e.g.*, Biorad Gel Doc XRS+ Imaging System, catalog number: 1708265)

Procedure

A. Generation of stable cell lines for the expression of recombinant CD89

If using commercial CD89 from Sino Biological, skip sections A and B, and go directly to section C.

1. To generate recombinant soluble CD89 protein (rCD89), synthesize DNA that encodes human

FcαRI/CD89 ectodomain (Met^{1}-Asn^{227} based on RefSeq accession: NP_001991.1) fused to a C-terminus 3×Flag of DYKDHD-G-DYKDHD-I-DYKDDDDK sequence.

2. Clone the cDNA fragment into pcDNA3 vector for expression using mammalian cells.
3. Transfect pcDNA3-rCD89-Flag plasmid into HEK293 cells cultured in growth medium using Lipofectamine 2000 in 6-well plates following the standard transfection protocol. The plasmid (2.5 μg, diluted in 100 μL of Opti-MEM medium) and Lipofectamine 2000 (7.5 μL, diluted in 100 μL of Opti-MEM medium) are mixed and incubated at room temperature for 15 min, then added to the well and mixed with the culture medium by gently pipetting.
4. After one day, dissociate the transfected cells by trypsin digestion and transfer the cells to 100 mm cell culture dishes with five-fold serial dilutions (five 100 mm dishes are used, with dilution ranges from 1:5 to 1:3125). Maintain the cells in 15 mL of selection medium.
5. Replace the selection medium every 3 days for 14 days until distinct cell clones are formed with an average of 100–500 cells per clone.
6. Isolate well-formed individual stable cell colonies under a microscope using a 20 μL pipette (by gently scratching and then catching the detached cell clones) and seed them in 96-well plates.
7. Maintain the cells in the selection medium for 5–7 days without changing the medium. Test the medium in each well for recombinant expression of rCD89-Flag by dot-blotting using HRP-conjugated anti-DDDDK tag antibody.
    a. Prepare a strip of nitrocellulose membrane and draw grids (1 cm × 1 cm) on it with a pencil.
    b. Spot 5 μL of medium samples onto the nitrocellulose membrane at the center of the grids. Minimize the area that the samples penetrate (usually less than 4 mm in diameter) by applying it slowly. Wait until the membrane is dry.
    c. Block the membrane with 5% dry milk in TBST and incubate for 1 h at room temperature.
    d. Pour off the block buffer, and keep the membrane always wet for the remainder of the procedure.
    e. Incubate the membrane with HRP-conjugated anti-DDDDK tag antibody (dilute at the ratio of 1:1,000 with 2% non-fat milk in TBST) for 1 h at room temperature.
    f. Wash the membrane three times with TBST.
    g. Incubate the membrane with ECL reagent and image with a chemiluminescence imaging system. The ECL reagent detects protein at the picogram level.
8. Select 2–3 stable cell clones with the highest recombinant protein expression and expend these individual clones to culture in 100 mm dishes.
9. Freeze down stable cell clones with cryopreservation medium (2 × 10^{7} cells/mL) using a cell freezing container (*e.g.*, Nalgene 5100-0036), and then store the cell bank in a liquid nitrogen tank.

B. Expression and purification of recombinant CD89-Flag

1. Culture a high-yielding stable cell clone in 100 mm dishes with DMEM supplemented with 10% FBS and 500 μg/mL G418.
2. When the cells grow to ~90% confluency, change the medium to serum-free DMEM and culture for another 5 days to allow the production of rCD89-Flag protein in the culture medium.
3. Harvest the medium (~100 mL) and then concentrate the medium with Amicon Ultra-15 filters with a molecular weight cut-off of 10,000 Da. Spin the filters at 4,000 × *g* at room temperature until the sample was concentrated to less than 5 mL.
4. Assemble a 5 mL gravity column packed with 2 mL of anti-flag M2 antibody-conjugated affinity gel. Load the concentrated medium into the column.
5. Wash the gravity column three times with PBS (30 mL each time). Then elute the recombinant protein with 5 mL of 3× Flag peptides at 150 μg/mL in PBS.
6. Collect the elution buffer containing the rCD89 protein, and dialyze the purified protein against PBS buffer overnight at 4°C by using Slide-A-Lyzer Dialysis Cassettes with a molecular weight cut-off of 10,000 Da.

7. Measure the total protein concentration using Bio-Rad protein assay reagent, following the manufacturer's instruction.
8. Store the recombinant protein at -80°C.

C. Detection of poly-IgA complexes in plasma by performing rCD89-directed ELISA

1. Plate Preparation
   a. Dilute rCD89 protein in carbonate sodium buffer (pH = 9.6) to a final concentration of 10 μg/mL. Immediately coat a 96-well ELISA microplate or strips with 100 μL of rCD89 protein per well. Seal the plate and incubate the reactions overnight at 4°C.
   b. Aspirate each well and then wash the wells with 300 μL of washing buffer. Repeat the process two more times for a total of three rounds of washes. After the last wash, remove any remaining wash buffer by aspiration, and then by inverting the plate to be patted against a stack of clean paper towels.
   c. Block the plate by adding 300 μL of blocking buffer to each well. Incubate the blocking reactions at 37°C for 1 h.
   d. Repeat aspirating and washing cycles as described in step C1b. The plates are now ready for measuring samples. However, avoid letting the empty wells to get air dried for a long time.
2. Prepare plasma samples and IgA standard
   a. Dilute the plasma samples 1:1,000 in blocking buffer (Plasma can be from freshly harvested samples, or from frozen stocks retrieved from -80°C storage. Based on our test results, 2–3 rounds of freeze-thaw cycles with storage at -80°C do not significantly change the reading of poly-IgA contents using the assay. If there is any precipitation from the freeze-thaw process, plasma samples should be centrifuged to remove the precipitates at 3000 x g at 4°C for 15 min).
   b. Dilute purified human IgA1 standard in blocking buffer in a concentration series ranging from 500 μg/mL to 7.8125 μg/mL.
3. Assay procedure
   a. Add 100 μL of diluted plasma samples or IgA1 standards in blocking buffer to each well. Cover the plate with an adhesive strip and incubate the reactions for 3 h at 37°C.
   b. Perform aspiration/washing cycles as described in step C1b above.
   c. Dilute the detection antibody (HRP-conjugated anti-human IgA antibody) 1:1,000 in blocking buffer. Add 100 μL of detection antibody solution to each well. Cover the plate with a new adhesive strip and incubate the reactions for 1 h at 37°C.
   d. Wash the wells three times as described in step C1b.
   e. Add 100 μL of TMB substrate solution to each well. Incubate the reactions for 25 min at room temperature. Avoid placing the plate under direct light.
   f. Add 100 μL of stop solution to each well.
   g. Measure the optical density of each well using a microplate reader under dual-wavelength set at 450/570 nm.

Data analysis

1. For testing plate-to-plate variability, in addition to the human IgA1 standard series, a sample standard is also included on each plate as the quality control. Only when the coefficient of variation of the quality control between different test plates is less than 10% the test optical density value is considered stable and reliable.
2. Concentrations of measured poly-IgA complexes in plasma for each sample are estimated by four-parameter logistic equation:
   [y = (A - D) / [1 + (x/C) ^ B] + D
   where y = optical density value, x = concentration, A = minimum asymptote, B = slope factor, C = concentration corresponding to the response midway between A and D, and D = maximum asymptote] (Figure 2) (Zhang *et al.*, 2021)].

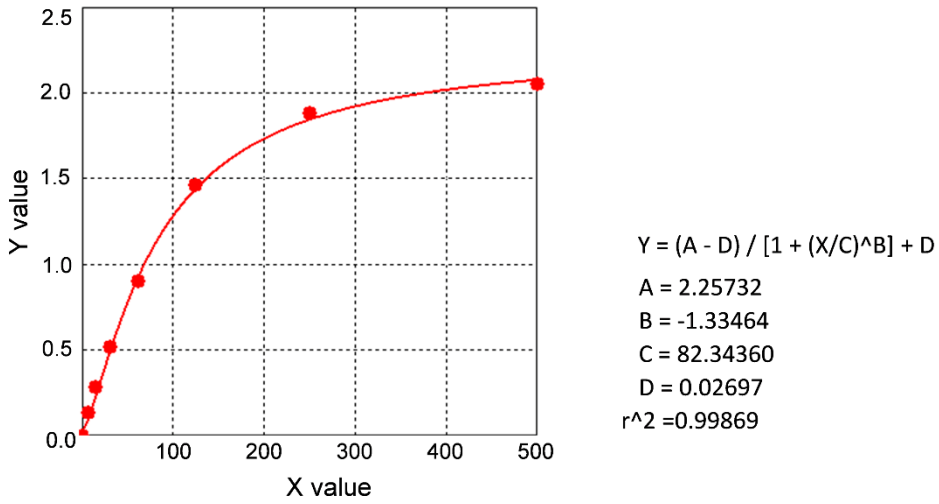


Figure 2. The standard curve for determining the plasma concentration of poly-IgA complex as measured by rCD89-directed ELISA.
The *y*-axis values are readings of the optical density of the TMB reactions at 450 nm. The *x*-axis values are marked as the corresponding concentrations of the human IgA1 standards (μg/mL). The data series (the red dots) is obtained from measurements of the human IgA1 standards between 7.8125 and 500 μg/mL. The fitting curve is derived from the four-parameter logistic regression model, which is subsequently used to calculate the unit concentration of poly-IgA complex in patient samples.

3. In this protocol, the concentration unit of poly-IgA complex is indexed as U/mL that is equally converted from the μg/mL value of measured IgA. The reason for using U/mL here instead of μg/mL is to clarify the measured content being poly-IgA, as opposed to total IgA.

Recipes

1. Growth medium
Dulbecco's Modified Eagle's Medium (DMEM)
10% fetal bovine serum (FBS)
100 U/mL penicillin and streptomycin

2. Selection medium
Growth medium supplemented with G418 to 1 mg/mL.

3. Cryopreservation medium
Growth medium supplemented with DMSO to a final concentration of 10%.

4. 0.05 M carbonate sodium buffer (pH 9.6)
a. Deposit one tablet in a beaker placed on a magnetic stirrer.
b. Add 50 mL of deionized water and stir the solution for a few min.
c. Adjust the water up to 100 mL, stir until full dissolution, and the buffer is ready to use.

5. 0.01 M Phosphate Buffered Saline (PBS) buffer
a. Dissolve a 1 L bag of PBS powder in 500 mL of deionized water.
b. Adjust the water up to 1,000 mL, stir until full dissolution, and the buffer is ready to use.

6. ELISA washing buffer (0.1% PBST buffer, pH 7.4)

Reagent	Final concentration	Amount
0.01 M PBS buffer	0.01M	999 mL
Tween-20	n/a	1 mL
Total	n/a	1,000 mL

7. ELISA blocking buffer (1% BSA/PBST buffer)

Reagent	Final concentration	Amount
Bovine serum albumin	1%	1g
0.1%PBST buffer	0.1%	100 mL
Total	n/a	100 mL

* Dissolve BSA in PBST buffer by gently rocking the capped container.

8. ELISA stop solution (1 M H_2SO_4)

Reagent	Final concentration	Amount
98% concentrated sulfuric acid	1 M	27.8 mL
ddH_2O	0.1%	472.2 mL
Total	n/a	500 mL

Acknowledgments

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Competing interests

J. Jin reports having an ownership interest in Accubit Inc.; being an advisor to Shenzhen Lujing Biotechnology Corporation, Limited, which is developing this technology toward clinical application in diagnosis; having consultancy agreements with Alebund Pharmaceuticals, Mannin Research Inc., and Qbio Med, Inc.; and serving as a scientific advisor for, or member of, Scientific Reports. J. Jin, J. Lv, and H. Zhang report applying for a patent related to the methodology described in this protocol for measuring the level of poly-IgA complex in clinical samples. H. Zhang reports serving as a vice-director of the nephrology committee in the Beijing Society of Medicine, board committee member of nephrology in the Chinese Medical Doctor Association, board committee member of the Chinese Society of Nephrology, member of the International Society of Nephrology (ISN)–Advancing Clinical Trials committee and member of ISN Sister Renal Centers Global Outreach committee and having consultancy agreements with Calliditas, Janssen, Novartis, and OMEROS. X. Li is an employee of Shenzhen Lujing Biotechnology Corporation, Limited. All remaining authors have nothing to disclose.

Ethics

The research was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the local ethics committees.

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