

Human Nephrin ELISA

Dilute Standards and Samples

Add to Wells

Add Anti-hu Nephrin Antibody

Incubate Overnight

Wash Plate

Add Conjugate

Incubate for 2 hours

Wash Plate

Develop for 5-20 Minutes

Add Color Stopper



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Human Nephrin ELISA: An ELISA designed to measure human urinary nephrin.

Intended Use: Human Nephrin ELISA is a competitive ELISA for the measurement of human Nephrin excretion. It is for research purposes, and is not intended for diagnostic use.

Technical Background: The transmembrane protein nephrin is expressed in renal glomerular podocytes, the visceral epithelial cells that line the outer aspect of the glomerular basement membrane (1-5). Podocytes contain interdigitated foot processes constituting a slit diaphragm that regulates the passage of plasma proteins across the glomerular filtration barrier. Mutation of the nephrin gene, altered nephrin production and abnormal podocyte function lead to proteinuria in diseases affecting the glomerulus such as nephrotic syndrome and diabetes. (1,6-8). Podocyte injury may be accompanied by shedding of the nephrin protein and/or of podocytes into the urine, and studies in experimental animals and in human diabetes support the hypothesis that nephrinuria is a marker for, and may reflect severity of, glomerular filtration dysfunction (9-11). ELISA measurement of urinary nephrin may provide an avenue for detection of early renal dysfunction and/or for assessing response to therapeutic interventions in experimental animal and clinical research.

Human Nephrin ELISA uses human nephrin as a standard, and a mouse antibody raised against the N-terminal portion of human nephrin. This antibody does not react with human albumin in this ELISA or Western blot. The assay is conducted in an indirect competitive mode and results are reported in ng/ml human nephrin.

Human Nephrin ELISA plates are coated with a preparation of human nephrin. To complete the assay, diluted standard and samples are added to respective wells. The mouse anti-human nephrin antibody is added. The antibody interacts and binds with the nephrin immobilized to the stationary phase or with that in the fluid phase, hence the notion of competitive binding. A subsequent reaction with anti-mouse IgG-HRP conjugate labels the probe with enzyme.

After washing, only the antibody-conjugate reacting with the anti-nephrin antibody bound to

nephrin antigen of the stationary phase remains in the well, and this is detected using a chromogenic reaction. Color intensity in Nephrin ELISA is inversely proportional to the logarithm of nephrin concentration in the fluid phase.

Specimen Collection and Storage: Collect samples without preservative. Store urine at -60°C to -80°C for up to 2 months. Prior to assay, allow the samples to come to room temperature. Do not apply heat to thaw frozen samples.

Kit Contents: The Human Nephrin ELISA kit contains the following items:

- a. 1 Hu-Nephrin Assay Plate
- b. 2 EIA diluent
- c. 1 Hu-Nephrin Standard
- d. 1 Mouse Anti-Nephrin antibody
- e. 1 Anti-mouse IgG-HRP Conjugate
- f. 1 Color Developer
- g. 1 Color Stopper
- h. 1 Instructions

Hu-Nephrin Standard, EIA diluent, Mouse anti-hu-nephin Antibody and Anti-mouse IgG-HRP Conjugate preparations contain 0.05% Proclin 300 (active components isothiazolones) as preservative. Color Stopper contains dilute (2.0 N) sulfuric acid. Save all unused reagents for future assays with this kit.

Human Nephrin ELISA plates are precoated and ready to use. All kit reagents are supplied in ready to use liquid form. A provision to wash the plates should be made. An EIA Wash Buffer with composition: 0.15 M NaCl, 0.01 M triethanolamine (pH 6.8), 0.05% Tween 20 and 0.05 % Proclin 300 (preservative may be omitted if the buffer is freshly prepared) is recommended.

Micropipettors capable of delivering 10, 50, 100 and 120 uL are required. Multi-channel pipettors capable of delivering 50 and 100 uL are recommended. In addition, small test tubes are required to complete dilutions (microfuge tubes work well in this application). Finally, a microplate reader equipped to determine absorbance at 450 nm is required.

Assay Procedure: Allow reagents and samples to come to room temperature before running the assay. The assay performs better when room temperature is above 24°C.

These instructions are written with the intent to complete the assay using duplicate wells for each dilution of standard, and each dilution of sample. The Human Nephrin ELISA may be used to determine the concentrations of up to 40 samples if performed at a single dilution.

Standard Dilutions: The standard is supplied as a two-fold concentrate. This procedure describes the preparation of seven (7) two-fold dilutions of standard.

1. Prepare 7 microfuge tubes with 120 uL of EIA diluent per tube.
2. Label the tubes numbers 1-7.
3. Transfer 120 uL of Hu-Nephrin Standard to tube 1.
4. Mix contents by aspirating and expelling the fluids 5 times.
5. Transfer 120 uL of solution from tube 1 to tube 2.
6. Mix as before.
7. Continue this procedure through tube number 7.
8. Tubes 1-7 now contain dilutions representing 2, 1, 0.5, 0.25, 0.12, 0.06, and 0.03 ug/ml human nephrin.

Preparation of Urine Sample Dilutions: Nephrin concentrations in urine from normal and diseased patients may vary and collection methods as well as kidney function (or dysfunction) may lead to very high or low concentrations. A starting dilution of 1:5 for specimens is suggested. For initial studies, and particularly if both normal and diseased samples are represented, it is wise to complete the analysis at more than one dilution, for example 1:5, 1:10, 1:20. It is recommended that sample dilutions be performed in tubes; dilution in the plate is not recommended.

After thawing, mix the urine samples and let them stand at room temperature for at least 15 minutes for particulates to settle down to the bottom of the tube.

Addition of Controls, Standard Nephtrin Dilutions and Samples to the plate: Label the strips of the plate with an indelible marker 1-12 (or A-H as required). This will allow reconstruction of the plate should strips fall out during the washing procedures.

The diluted standards and samples may be added directly to the dry plate. The plate design described here includes two controls: a negative control termed C0, and a positive one termed C1. These are placed in wells A1 and A2 respectively. All other wells receive either 50 ul of diluted standard or 50 ul of diluted sample.

1. Add 100 uL EIA diluent from the stock bottle to well A1. This is the negative control "C0" and will be used to standardize or "blank" the microplate reader.
2. Add 50 uL EIA diluent to well A2. This is the positive control "C1" and is a qualitative indicator of assay performance.
3. With a fresh tip, pre-wet the tip in nephtrin dilution 1, and transfer 50 uL aliquots to wells B1 and B2.
4. With a fresh tip, pre-wet the tip in dilution 2, and transfer 50 uL aliquots to wells C1 and C2.
5. Continue transferring diluted standard to the plate in this fashion, i.e. in order, taking care to pre-wet the tip in the new dilution each time.
6. Using a new tip, pre-wet the tip, and add 50 uL aliquots of diluted sample to wells A3 and A4.
7. Continue adding diluted samples to the plate, taking care to change the tip for each one.
8. The plate now contains controls, standard dilutions, and diluted experimental samples in duplicate in the balance of the plate.

Primary Incubation: Reaction with Mouse Anti-Hu-Nephtrin Antibody

1. Add 50 uL of Mouse anti-Hu-Nephtrin Antibody to Wells A2-A12, and B-H 1-12. Do not add antibody to well A1.
2. Cover and incubate the plate overnight at room temperature.

Secondary Incubation: Reaction with Anti-mouse IgG-HRP Conjugate:

1. Use a plate washer or wash plates by hand as follows:
 - a. Remove fluids from the well, i.e. aspirate off fluids or flip them out into a sink.
 - b. Fill wells to over-flowing with the recommended wash buffer.
 - c. Remove fluids as before.
 - d. "b" and "c" constitute a wash cycle.
 - e. Repeat the process to yield a total of 3 wash cycles.
 - f. Invert the plate on a paper towel and tap gently to remove adherent fluids.

2. Add 100 uL of Anti-mouse IgG-HRP Conjugate to every well on the plate.
3. Incubate as before for 2 hours.

Color Development:

1. Wash Plate as above for 5 wash cycles.
2. Add 100 uL of Color Developer to each well.
3. Develop 5- 20 minutes.
4. Add 100 uL of Color Stopper to each well.
5. Use a plate reader to determine the absorbance of experimental wells at 450 nm blanked against well A1.

Analysis: This analysis assumes that computer and analysis software is available, i.e. Excel.

1. Prepare a spreadsheet entering appropriate data including standard dilution, concentration, sample dilution and absorbance data. Determine the mean for replicate wells.
2. Prepare a semi-logarithmic plot of standard dilutions with the log [Nephtrin] on the x-axis and the mean absorbance on the y-axis.
3. The data that fall into the linear portion of the dose-response curve constitute the usable portion of the assay.

4. Subject these data to semi-logarithmic analysis to yield a mathematical model, of the form:

$$\log_{10} [\text{Nephtrin}] = m A_{450} + b$$

5. Nephtrin concentration is determined by taking the anti-log of the calculated values from this equation.
6. Multiply by reciprocal of dilution to determine the concentration of undiluted samples.

Quality Control: Record Keeping: It is good laboratory practice to record the lot numbers and dates of the kit components and reagents for each assay.

Sample Handling: The samples should be secured, processed and stored as discussed above.

Dilute Standard and Samples carefully. For the standards, a single tip may be used to prepare the dilution series. For experimental samples a fresh tip should be used for each urine specimen.

Limitations: It is the responsibility of the investigator to determine if the presence of experimental compounds or their metabolites in the urine will affect the assay results. Gross microbiological contamination may affect assay results.

Trouble Shooting:

1. No color appears after adding Color Developer: One or more reagents may have been adversely affected by storage above 8°C. One or more reagents may not have been added. Repeat assay. Be sure to store the kit appropriately.
2. Color in wells too light: Longer incubation with Color Developer may be required. If the color is still too light after 20 minutes development, repeat the assay but increase the secondary incubations to 3 hour.
3. Color in wells is too dark: Decrease the development time. If a 5 minute development is still too dark, repeat the assay and reduce the secondary incubation to 90 minutes. If color is dark and the standard dilutions fail to show the appropriate dose-response, Color Developer may have been contaminated with conjugate or the plate was poorly washed. Repeat the assay and take care in the pipetting and in the washing operations.
4. Color in sample well(s) is darker or lighter than lowest or highest concentrations of the standard curve. Change sample dilution protocol appropriately.
5. Poor agreement between duplicate wells: This is almost always due to pipetting error. Repeat the assay.

6. Microplate ELISAs may be prone to edge effects wherein the outer rows and columns show a darker response than the inner ones. This effect may be minimized by incubating the plate in a closed humid container.

A plastic food storage container with a tight fitting lid and a water moistened paper towel work well in this respect. Place the moistened towel in the bottom of the container, and place the plate upon it. Position the cover and incubate as described.

References:

1. Putaala, H et al: The murine nephtrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and death. *Hum Mol Genet* 10:1-8, 2001
2. Putaala, H. et al: Primary structure of mouse and rat nephtrin cDNA and structure and expression of the mouse gene. *J Am Soc Nephrol* 11:991-1001, 2000
3. Holtzman, LB et al: Nephtrin localizes to the slit pore of the glomerular epithelial cell. *Kidney Int* 56:1481-1491, 1999
4. Routsalainen, V et al: Nephtrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci USA* 96:7962-7967, 1999
5. Holthofer, H et al: Nephtrin localizes at the podocyte filtration area and is characteristically spliced in the human kidney. *Am J Pathol* 155:1681-1687, 1999
6. Kestila, M et al: Positionally cloned gene for novel glomerular protein – nephtrin – is mutated in congenital nephrotic syndrome. *Mol Cell* 1:575-582, 1998
7. Cooper, ME et al: Role of nephtrin in renal disease including diabetic nephropathy. *Semin Nephrol* 22:393-398, 2002
8. Pagtalunan, ME et al: Podocyte loss and progressive glomerular injury in type 2 diabetes. *J Clin Invest* 99:342-348, 1997
9. Aaltonen, P et al: Changes in the expression of nephtrin gene and protein in experimental diabetic nephropathy. *Lab Invest* 81:1185-1190, 2001
10. Luimula, P et al: Nephtrin in experimental glomerular disease. *Kidney Int* 58:1461-1468, 2000
11. Patari, S et al: Nephtrinuria in diabetic nephropathy of type 1 diabetes. *Diabetes* 52:2969-2974, 2003

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