Nephrin ELISA

Dilute Standards and Samples

Add to Wells

Add Anti-Nephrin Antibody

Incubate for 60 Minutes

Wash Plate

Add Conjugate

Incubate for 60 Minutes

Wash Plate

Develop for 5-20 Minutes

Add Color Stopper

Less than 2 ½ hours to complete

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Nephrin ELISA: An ELISA designed to measure urinary nephrin

Intended Use: Nephrin ELISA is a competitive ELISA for the measurement of 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.

Nephrin ELISA uses rat nephrin in a urine matrix as a standard, and rabbit polyclonal antibody raised against the N-terminal portion of human nephrin that cross-reacts with nephrin of several mammalian species, particularly human, rat and mouse. The assay is conducted in an indirect competitive mode and results are reported in ug rat nephrin equivalents.

Nephrin ELISA plates are coated with a preparation of rat nephrin. To complete the assay, diluted standard and samples are added to respective wells. The anti-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-rabbit -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 porportional to the logarithm of nephrin concentration in the fluid phase.

Specimen Collection and Storage: Collect samples without preservative, and clarify them by centrifugation if necessary. Store clarified urine at 4^{o}C for up to 1 week or at -60^{o}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 Nephrin ELISA kit contains the following items:

a. 1 Nephrin Assay Plates
b. 2 NHEBSA diluent
c. 1 Nephrin Standard
d. 1 Rabbit Anti-Nephrin antibody
e. 1 Anti-rabbit HRP Conjugate
f. 1 Color Developer
g. 1 Color Stopper
h. 1 Instructions

Nephrin Standard, NHEBSA diluent, Rabbit anti-nephrin Antibody and Anti-rabbit 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.

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. Tap water has been shown suitable in experimental and quality control contexts, but 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^{o}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 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 nine (9) two-fold dilution of standard. It is possible to extend the curve another 2 dilutions, if needed.

1. Prepare 9 microfuge tubes with 120 uL of NHEBSA per tube.
2. Label the tubes numbers 1-9.
3. Transfer 120 uL of 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 9.
8. Tubes 1-9 now contain dilutions representing 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, and 0.008 ug rat equivalents/ml.

Preparation of Urine Sample Dilutions: Nephrin concentrations in urine from normal and diseased animals may vary and collection methods as well as kidney function (or dysfunction) may lead to very high or low concentrations. A starting dilution of 1:10 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 1:10, 1:20, 1:40.

It is recommended that sample dilutions be performed in tubes; dilution in the plate is not recommended.

Addition of Controls, Standard Nephrin 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 diluted standard or diluted sample. The assay volume is 50 uL.

1. Add 100 uL NHEBSA 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 NHEBSA 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 nephrin 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 C3 and C4.
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 Rabbit Anti-Nephrin Antibody

1. Add 50 uL of Rabbit Anti-Nephrin Antibody to Wells A2-A12, and B-H 1-12.
2. Cover and incubate the plate for 60 minutes at room temperature.

Secondary Incubation: Reaction with Anti-rabbit 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 10 wash cycles.
f. Invert the plate on a paper towel and tap gently to remove adherent fluids.

2. Add 100 uL of Anti-rabbit HRP Conjugate to every well on the plate.
3. Incubate as before for 60 minutes.

Color Development:

1. Wash Plate as above
2. Add 100 uL of Color Developer to each well.
3. Develop 5- 10 minutes
4. Add 100 uL of Color Stopper to each well.

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

1. Use a plate reader to determine the absorbance of experimental wells at 450 nm blanked against well A1.
2. Prepare a spreadsheet entering appropriate data including standard dilution, concentration, sample dilution and absorbance data. Determine the mean for replicate wells.
3. Prepare a semi-logarithmic plot of standard dilutions with the log [Nephrin] on the x-axis and the mean absorbance on the y-axis.
4. The data that fall into the linear portion of the dose-response curve constitute the usable portion of the assay.

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

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

6. Nephrin concentration is determined by taking the anti-log of the calculated values from this equation.

7. 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. Rodent urine is often contaminated by food and fecal material, and these contaminants present potential sources of error. Centrifugation to clarify samples is recommended.

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 adversly affected by storage above 8ºC. 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 10 minutes development, repeat the assay but increase the primary and secondary incubations to 2 hour each.
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 30 minutes.
   If color is dark and the standard dilutions fail to show the appropriate dose-response, Color Developer may have been contaiminated 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.

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