Nephrin R ELISA

Competitive assay for the quantitative determination of rat urinary nephrin.

- Dilute Standards and Samples
- Add to Wells
- Add Anti-Rat Nephrin Antibody
- Incubate Overnight
- Wash Plate
- Add Conjugate
- Incubate for 120 Minutes
- Wash Plate
- Develop for 5-20 Minutes
- Add Color Stopper

Exocell: Division of Ethos Biosciences, Inc.
1880 JFK Blvd.
Suite 200
Philadelphia, PA 19103

Phone: (215)557-8021
Fax: (215)557-8053
Email: exocell@verizon.net
http://www.exocell.com

Nephrin R ELISA: An ELISA designed to measure rat urinary nephrin.

Intended Use: Nephrin R ELISA is a competitive ELISA for the measurement of rat 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; 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 and clinical research.

Nephrin R ELISA uses rat nephrin as a standard (Rat Nephrin Standard), and a mouse antibody raised against the N-terminal portion of rat nephrin (Anti-Rat Nephrin AB). This antibody does not react with rat albumin in this ELISA or on Western Blot. The assay is conducted in an indirect competitive mode and results are reported in ug/ml rat nephrin.

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

.

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

a. 1 Rat Nephrin Assay Plate
b. 2 EIA Diluent
c. 1 Rat Nephrin Standard
d. 1 Anti-Rat Nephrin AB (Antibody)
e. 1 Anti-Mouse IgG HRP Conjugate
f. 1 TMB Color Developer
g. 1 Acid Color Stopper
h. 1 Instructions

Rat Nephrin Standard, EIA Diluent, Anti-Rat Nephrin AB 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.

Rat Nephrin Assay Plates are precoated and ready to use. All kit reagents are supplied in ready to use liquid form.

Other Materials and Solutions Recommended but not provided:

EIA Wash Buffer: 0.15 M NaCl, 0.01 M triethanolamine (pH 6.8), 0.05% Tween 20.

Micropipettors and tips capable of delivering 10, 50, 100 and 120 uL are required.
Multi-channel pipettors capable of delivering 50 and 100 uL are recommended.

Microfuge test tubes for dilution of standard and samples are required.

Microplate reader equipped to determine absorbance at 450 nm is required.

Specimen Collection and Storage: Collect samples without preservative. Store urine at -60°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

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.

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.

Standard and Samples, once at room temperature, should be gently mixed then allowed to sit for a minimum of 15 minutes to allow any particulates to settle.

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 R 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 dilution 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 Rat 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 10.0, 5.0, 2.5, 1.25, 0.625, 0.313, 0.156 ug/ml rat nephrin..

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 1:5 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.

Addition of Controls, Standard Nephrin Dilutions and Samples to the plate: Label the strips of the plate with an indelible marker 1-12. 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 standard and/or sample assay volume is 50 uL per well.

1. Add 100 uL EIA DILUENT 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 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 Standard 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 wellsC1 and C2.
5. Continue transferring diluted standard to the plate in this fashion, i.e. in order through H1 and H2, 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 **Anti-Rat Nephrin AB**. This is the primary antibody.`

1. **Do not add Anti-Rat Nephrin AB to well A1.** Add 50 uL of Anti-Rat Nephrin AB to A2 and to all remaining wells.
2. Cover and incubate the plate overnight at room temperature in a moist chamber.

Secondary Incubation: Reaction **with Anti-Mouse IgG HRP Conjugate**:

1. Aspirate each well and wash with EIA Wash Buffer repeating the process two times for a total of three cycles. Wash by filling each well with 400 uL using a squirt bottle or auto-plate washer. Complete removal of liquid after each cycle is essential for good performance: Invert the plate on a clean paper towels and tap gently to blot any adherent fluids.
2. Add 100 uL of Anti-mouse IgG-HRP Conjugate to every well on the plate.
3. Cover and incubate the plate for 120 minutes at room temperature.

Color Development:

1. Wash Plate as Step 1, 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 and record the absorbance of all experimental wells at 450 nm, blanked against well A1.

Analysis:

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

Prepare a spreadsheet entering appropriate data including standard dilution, concentration, sample dilution and absorbance data. Determine the mean for replicate wells.

Prepare a semi-logarithmic plot of standard dilutions with the log [Nephrin] on the x-axis and the mean absorbance on the y-axis.

The data that fall into the linear portion of the dose-response curve constitute the usable portion of the assay.

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

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

Nephrin concentration is determined by taking the anti-log of the calculated values from this equation. Multiply by the dilution factor of the sample to determine the concentration of undilute sample.

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.

Trouble Shooting:

1. No color appears after adding Color Developer: One or more reagents may not have been added. One or more reagents may have been adversly affected by storage above $8^{o}C$. 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 hours.
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 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.

References:

1. Putaala, H et al: The murine nephrin 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 nephrin cDNA and structure and expression of the mouse gene. J Am Soc Nephrol 11:991-1001, 2000

3. Holtzman, LB et al: Nephrin localizes to the slit pore of the glomerular epithelial cell. Kidney Int 56:1481-1491, 1999

4. Routsalainen, V et al: Nephrin is specifically located at the slit diaphragm of glomerular podocytes. Proc Natl Acad Sci USA 96:7962-7967, 1999

5. Holthofer, H et al: Nephrin 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 – nephrin – is mutated in congenital nephrotic syndrome. Mol Cell 1:575-582, 1998

7. Cooper, ME et al: Role of nephrin 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 nephrin gene and protein in experimental diabetic nephropathy. Lab Invest 81:1185-1190, 2001

10. Luimula, P et al: Nephrin in experimental glomerular disease. Kidney Int 58:1461-1468, 2000

11. Patari, S et al: Nephrinuria in diabetic nephropathy of type 1 diabetes. Diabetes 52:2969-2974, 2003

Nephrin R ELISA v 2.0: March, 2019

Disclaimer: Exocell makes no guarantee of any kind, expressed or implied, which extends beyond the description of the material in this kit, except that these materials and this kit will meet our specifications at the time of delivery. Customer's remedy and Exocell's sole liability hereunder is limited, at Exocell's option, to replacement of material that does not meet our specifications. By acceptance of our products, the customer indemnifies and holds Exocell harmless against, and assumes all liability for, the consequences of its use or misuse by the customer, its employees, or others.