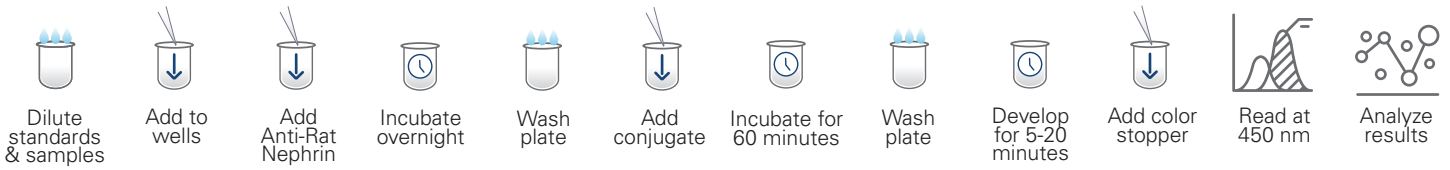
RAT NEPHRIN ELISA



DESCRIPTION

Our Rat Nephrin ELISA is a competitive ELISA for the measurement of rat nephrin excretion. It is intended for research purposes, but is not for diagnostic applications.

Technical Background: Nephrin is a transmembrane protein expressed in podocytes, the visceral epithelial cells that line the outer aspect of the glomerular basement membrane (1-5). These cells have interdigitating foot processes constituting slit diaphragms that regulate the passage of plasma proteins across the glomerular barrier, therefore performing a filtration function. Mutation of the nephrin gene, altered nephrin production, and abnormal podocyte function all lead to proteinuria in diseases affecting the glomerulus such as nephrotic syndrome and diabetes. (1,6-8). Kidney injury may be accompanied by release of nephrin and/or shedding of podocytes into the urine. Studies in animals and diabetic humans support the notion that nephrinuria is a marker for, and may reflect severity of, glomerular damage in kidney injury and disease (9-11). Measurement of urinary nephrin may enable early detection of renal dysfunction and/or assessment of renal response to therapeutic interventions.

Rat Nephrin 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 cross react with rat albumin in this ELISA or in Western Blot analysis. The assay is conducted in an indirect competitive mode and results are reported in ug/ml rat nephrin.

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

SPECIMEN COLLECTION AND STORAGE

Collect urine samples without preservative. Freeze and store 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.

KIT CONTENTS

- 1 Rat Nephrin Assay Plate
- 2 EIA Diluent
- 1 Rat Nephrin Standard
- 1 Anti-Rat Nephrin Ab (Antibody)
- 1 Anti-Mouse IgG HRP Conjugate
- 1 TMB Color Developer
- 1 Acid Color Stopper
- 1 Instructions

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

Materials and Equipment Needed, but Not Included in Kit

- Wash Buffer: Ethos Biosciences EIA Wash Buffer works well.
- Micropipettors tips capable of delivering 10, 50, 100, and 120 uL are required. Multi-channel pipettors capable of delivering 50 and 100 uL are recommended.
- Tubes for dilution (Microfuge tubes work well).
- Microplate reader equipped to measure absorbance at 450 nm is required.

Limitations

It is the responsibility of investigators to determine if Mouse Nephrin ELISA is suitable for use with their samples. The presence of experimental compounds or their metabolites in the urine and gross microbiological contamination may affect assay results.

Assay Procedure

Allow reagents to come to room temperature before running the assay.

Once at room temperature, samples and standards should be gently mixed and 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. This design allows analysis of up to 40 samples per plate.

Standard Dilutions: 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 2.0 – 0.0313 ug/ml rat nephrin.

Preparation of Urine Sample Dilutions: The goal is to dilute your samples into the range of the standard curve to obtain valid quantitative measurements. However, nephrin concentrations in urine from normal and diseased animals will vary due to collection methods and kidney function (or dysfunction). It is the responsibility of the researcher to assess their samples and appropriately dilute into the range of the standard curve. 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.

It is strongly recommended that sample dilutions be performed in tubes and not attempted in the plate.

Addition of Controls, Nephrin Standard 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 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 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 with Nephrin Standard dilution 1, and transfer 50 uL aliquots to wells B1 and B2.
4. With a fresh tip, pre-wet the tip with 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 through H1 and H2, taking care to pre-wet the tip with the new dilution each time.
6. Using a new tip, pre-wet the tip with diluted sample, 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 and pre-wet 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 primary Anti-Rat Nephrin Ab.

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. Wash the plate ten times (10X) with EIA Wash Buffer using a plate washer or manual methods. Flip out the fluids and blot dry after the final wash.
2. Add 100 uL of Anti-mouse IgG-HRP Conjugate from stock solution to every well on the plate.
3. Cover and incubate the plate for 60 minutes at room temperature.

Color Development:

1. Wash Plate as above, and blot after final wash.
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. Measure absorbance at 450 nm, blanking the plate reader on "C0" in well A1.

Analysis: This analysis assumes that computer and analysis

software is available, i.e. Excel. Prepare a spreadsheet entering a row for each standard or sample and a column for each of the following: dilution, standard concentration, log[std concentration], absorbance data and mean absorbance for each replicate. Determine the mean for replicate wells.

Prepare a (semi-logarithmic) standard curve of 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, log concentration and absorbance to regression analysis to yield a model of the form:

log10 [Nephrin] = m A450 + 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. For assistance with analysis, please contact customer service.

Quality Control: Record keeping: It is good laboratory practice to record lot numbers and expiration dates of the kit components 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

- "No color appears after adding Color Developer" One or more additions may have been omitted or reagents compromised by improper storage. Repeat assay.
- "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 double the secondary incubation time.
- "Color in wells is too dark" Decrease the development time. If a 5-minute development is still too dark; reduce secondary incubation by half.
- "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.
- "Color in sample well(s) is darker or lighter than lowest or highest concentrations of the standard curve" All samples need to fall within the standard curve to give valid quantitative measurement data. If a sample is darker than the darkest standard, it is too dilute. If it is lighter than the lightest standard it is too concentrated. Repeat assay and dilute appropriately.
- "Poor agreement between duplicate wells" There is most likely pipetting error. Repeat the assay and ensure precise pipetting and proper dilution.
- Hint: Microplate ELISAs may be prone to edge effects wherein the wells show a darker response than the inner ones due to evaporation. This effect may be minimized by incubating the plate in a closed humid chamber. A plastic or glass container with sealable lid works well. Place a water moistened paper towel in the bottom of the container, and place the plate upon it. Close the cover and incubate as described.
- Hint: In a competitive ELISA antibody binding occurs with both fluid borne and surface bound antigen. In dilute samples little antigen is present in the fluid phase, and most of the antibody binds to the surface bound antigen. This leads to a dark color upon development. In contrast, at high antigen concentrations, most of the antibody binds to antigen in the mobile phase; leaving little to bind to the antigen on the solid phase. Subsequent reactions and color development produce lighter color. Therefore; color intensity in a competitive ELISA is inversely proportional to the log of antigen concentration.
- Hint: There is variability of urinary nephrin due to animal, treatment and collection protocol. Nephrin measurements may be normalized using creatinine concentration measured in the same sample.

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PRODUCT INFORMATION

Cat. #	Description
1036	Rat Nephrin ELISA

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