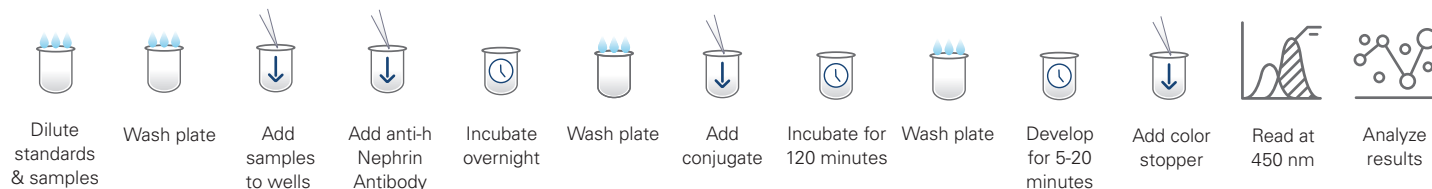


HUMAN NEPHRIN ELISA



DESCRIPTION

Human Nephtrin ELISA is a competitive ELISA for the measurement of nephtrin in human urine specimens. It is intended for research purposes.

Technical Background: Nephtrin 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 nephtrin gene, altered nephtrin 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 nephtrin and/or shedding of podocytes into the urine. Studies in animals and diabetic humans support the notion that nephtrinuria is a marker for, and may reflect severity of, glomerular damage in kidney injury and disease (9-11). Measurement of urinary nephtrin may enable early detection of renal dysfunction and/or assessment of renal response to therapeutic interventions.

Human Nephtrin ELISA uses human nephtrin (h nephtrin) as a standard, and a mouse monoclonal antibody raised against the N-terminal portion of human nephtrin. This antibody does not cross-react with human albumin.

Human Nephtrin ELISA is an indirect competitive ELISA, and results are reported in $\mu\text{g}/\text{ml}$ h nephtrin. The plates are coated with h nephtrin. Diluted standard and samples are added to respective wells.

The anti-h nephtrin antibody is added which binds with either the nephtrin immobilized to the stationary phase or with that in the fluid phase, hence the notion of competitive binding.

Unbound reactants are then washed away, and reaction with an anti-Mouse IgG HRP Conjugate is completed. The conjugate binds to antibody bound to the solid phase antigen; labeling it with enzyme.

After washing, a chromogenic substrate is added. After a short incubation period, the reaction is stopped with acid. The color intensity in the assay is inversely proportional to the logarithm of human nephtrin concentration in the initial 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. Samples should be mixed and allowed to sit a minimum of 15 minutes to allow particulates to settle.

KIT CONTENTS

- 1 Human Nephtrin Assay Plate
- 2 EIA Diluent
- 1 Human Nephtrin Standard
- 1 Mouse anti-Human Nephtrin MAb (antibody)
- 1 Anti-Mouse IgG HRP Conjugate

- 1 TMB Color Developer
- 1 Acid Color Stopper
- 1 Instructions

Human Nephtrin 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. PBS with 0.05% Tween 20 is also satisfactory. Water is unsatisfactory.
- Micropipettors and Multi-channel pipettors and tips
- Tubes for dilution (Microfuge tubes work well).
- Microplate reader equipped to measure absorbance at 450 nm is required.

LIMITATIONS

It is the responsibility of the investigator(s) to determine if Human Nephtrin 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.

Standard and Samples should be mixed gently then allowed to sit for a minimum of 15 minutes to allow 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 inclusion of controls, standard curve and provides for analysis of up to 40 samples.

Standard Dilutions

This procedure describes the preparation of seven (7) two-fold dilutions of standard.

1. Label the tubes numbers 1-7.
2. Add 120 uL of EIA DILUENT to each tube.
3. Transfer 120 uL of Human Nephtrin Standard to tube 1.
4. Mix contents by aspirating and expelling the fluids 5 times.
5. Transfer 120 uL of from tube 1 to tube 2.
6. Mix as before.
7. Continue doing this through tube number 7.
8. Tubes 1-7 now contain dilutions representing 2.0- 0.0313 ug/ml human nephtrin.

Preparation of Urine Sample Dilutions

Nephtrin concentrations in urine from normal and diseased

animals vary, and collection methods may affect concentration. A starting dilution of 1:5 for specimens is suggested, but 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, Standard Nephtrin Dilutions and Samples to the plate

Label the strips of the plate with an indelible marker 1-12. This will allow reassembly of the plate should strips fall out during the washing procedures.

WASH THE PLATE 5 X with wash buffer

THE PLATE MUST BE WASHED before the addition of controls, diluted standard or diluted samples. 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 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 Nephtrin 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 replace and 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 **Anti-Human Nephtrin MAb**

1. Do not add Anti-Human Nephtrin MAb to well A1.
2. Add 50 uL of Anti-Human Nephtrin MAb to A2 and to all remaining wells.
3. Incubate the plate overnight at room temperature in a moist chamber. A plastic food storage container works well. Place a water moistened paper towel in the bottom of the container, place the plate upon it and position the cover. Incubate as described.

Secondary Incubation: Reaction with **anti-Mouse IgG HRP-Conjugate Ab:**

1. Wash the plate five times with Wash Buffer.
2. Flip out the fluids, and blot dry after the final wash.
3. Add 100 uL of HRP-Conjugate Ab to each well.
4. Incubate the plate 2 hours at room temperature in a moist chamber.

Color Development

1. Wash Plate 5X as described above.
2. Flip out and blot after final wash.
3. Add 100 uL of Color Developer to each well.
4. Develop 5- 20 minutes at room temperature.
5. Add 100 uL of Color Stopper to each well.
6. Determine absorbance at 450 nm blanked against well A1.

Analysis: This analysis uses spreadsheet software such as Microsoft Excel.

Prepare a spreadsheet entering a row for each sample, and a column each for: Sample ID, dilution, standard concentration, log[std concentration], replicate absorbance data, mean absorbance, est Concentration and undilute concentration.

Enter standard/sample information and absorbance data, and determine the mean absorbance 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, log concentration and absorbance to regression analysis to yield a 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.

For assistance with analysis, please contact customer service.

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

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 is 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 range of the standard curve to give valid quantitative measurements. 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” This is most likely pipetting error. Repeat the assay and ensure precise pipetting and proper dilution.
- 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.

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: Nephrouria in diabetic nephropathy of type 1 diabetes. *Diabetes* 52:2969-2974, 2003

Ethos Biosciences, Inc. 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 Ethos Biosciences' sole liability hereunder is limited, at Ethos Biosciences' option, to replacement of material that does not meet our specifications. By acceptance of our products, the customer indemnifies and holds Ethos Biosciences, Inc. harmless against, and assumes all liability for, the consequences of its use or misuse by the customer, its employees, or others.

PRODUCT INFORMATION

Cat. #	Description
1035	Human Nephrin ELISA

Order today at EthosBiosciences.com

Trademarks: Excel® is a registered trademark of Microsoft, Tween® is a registered trademark of ICI Americas Inc, ProClin® is a registered trademark of Rohn and Haas Company, Exocell™ is a trademark of Ethos Biosciences,