

NEPHRAT II ELISA



DESCRIPTION

Exocell's™ Nephurat II is for the quantitative measurement of microalbuminuria in rat specimens.

Intended Use: Nephurat is a direct enzyme linked immunosorbent assay (ELISA) for the quantitative determination of albumin in rat urine. It is intended for research applications; it is not intended for diagnostic use.

Technical Background: Nephurat is intended for use as an in-vitro tool for assessing kidney function in rats. It is simple to perform and highly specific for rat albumin. It is a competitive antibody capture ELISA completed in a direct mode. To that end, the anti-albumin antibody (Ab) is conjugated to horseradish peroxidase (HRP), i.e. directly labeled.

To complete the assay, sample and anti-rat albumin Ab-HRP are added to mouse albumin-coated well. The antibody interacts and binds with the albumin immobilized to the stationary phase or with that in the fluid phase, hence the notion of competitive binding. Washing removes Ab-HRP- Albumin and unbound reactants of the fluid phase from the well. Only the antibody-HRP conjugate that was bound to the albumin of the stationary phase remains, and this is detected using Tetramethylbenzidine (TMB) in a chromogenic reaction. The reaction is stopped with acid, and absorbance is measured at 450 nm. Absorbance is inversely proportional to the logarithm of albumin in the fluid phase.

SPECIMEN COLLECTION AND STORAGE

Collect samples without preservative and clarify them by centrifugation if necessary. Store clarified urine at 4°C for up to 1 week or 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:

- 2 Assay Plates
- 2 NHEBSA (Diluent)
- 2 Rat Serum Albumin (RSA) Standard
- 2 Nephurat Conjugate
- 2 Color Developer
- 2 Color Stopper
- Instructions

RSA Standard, NHEBSA and anti-rat Antibody HRP preparations contain 0.05% Proclin 300 (active components isothiazolones) as preservative. Color Stopper contains dilute (2.0 N) sulfuric acid.

Nephurat assay plates are precoated and ready to use. All kit reagents are supplied in ready to use liquid form. A plate wash procedure using tap water has been shown to be suitable in experimental and quality control contexts. However, where tap water is unavailable or proves unsuitable; we recommend 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).

Micropipettors capable of delivering 10 & 100 uL are required. Multi-channel pipettors capable of delivering 100 uL

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ASSAY PROCEDURE

Please read the instructions, limitations and trouble shooting sections carefully before beginning the assay. It is recommended that samples be run in duplicate.

Urinary albumin varies significantly depending on kidney function (or dysfunction) and collection methods, and successful determination depends upon proper sample dilution. Accordingly, there are two procedures for completing analysis with Nephtrat: a **Basic Procedure** in which samples are diluted 1:10, and an **Alternate Procedure** in which they are serially diluted. Basic and Alternate procedures call for sample dilution directly in the Nephtrat Assay Plate.

The **Basic Procedure** results in a 1:10 dilution for all of the samples. It requires 20 μL of urine/sample to complete the analysis in duplicate, and 40 samples per plate may be analyzed.

The **Alternate Procedure** results in serially diluted samples beginning with a 1:2 dilution. In all 8 dilutions are completed per sample. Only 10 samples may be analyzed using this procedure.

Allow reagents and samples to come to room temperature before running the assay. Use only kit reagents and carefully follow instructions.

Standard Curve Preparation: Standard rat serum albumin (RSA) is serially diluted in two-fold dilutions to produce 10.0, 5.0, 2.5, 1.25, 0.625, 0.313, 0.156 and 0.0 mg/dL in duplicate wells. A standard curve must be run on each plate.

1. Place 100 μL of NHEBSA Diluent in each well in columns 1 and 2 (rows A-H) of the Nephtrat Assay Plate.
2. Transfer 100 μL of RSA each to wells A1 and A2.
3. Mix the fluids thoroughly by repeated pipetting.
4. Transfer 100 μL from wells A1 and A2 to wells B1 and B2 respectively.
5. Mix the fluids as described.
6. Continue this transfer/mix procedure through to wells G1 and G2. After mixture, remove/discard 100 μL from the wells.

Wells A1-G1 and A2-G2 have serially diluted RSA at 100 μL /well. Wells H1 and H2 have diluent in them, and represent 0 mg/mL.

Basic Procedure

Duplicate analysis of urine samples at 1:10 Dilution

1. Prepare a standard curve as described.
2. Add 90 μL of NHEBSA Diluent in the remaining columns 3-12, rows A-H.
3. Place 10 μL of urine from each sample into two adjacent wells (duplicates). Record the positions and dilution of each sample. All samples are now diluted 1:10.

Alternate Procedure

Eight dilutions of 100 μL urine for each of 10 samples

1. Prepare standard curve as described.
2. Add 100 μL of NHEBSA Diluent in the remaining wells of the plate (columns 3-12 and rows A-H).
3. Place 100 μL /well from each sample into row A of columns 3 - 12, using one column per sample. Mix the sample in the well using the pipet. Change pipet tips between samples, and record the sample positions.
4. Transfer 100 μL of the solutions in row A to row B. Repeat the mixing and transfer steps through row H. Withdraw and discard 100 μL from row H. Your samples will now be in twofold serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:64, 1:128 and 1:256 in rows A, B, C, D, E, F, G and H, respectively.

Incubation

All reagents should be at room temperature. After the plates are set up with both standards and samples, complete the following steps.

1. Add 100 μL Nephtrat Conjugate per well to all wells on the plate.
2. Incubate the plate at room temperature for at least 30 minutes. Longer incubations (up to one hour) produce acceptable results.

Color Development and Absorbance Determination

TMB Color Developer should be at room temperature.

1. Remove the fluids in the plate (i.e. flip them out into the sink)
2. Flood the plate with tap water or wash buffer (see kit contents section above).
3. Repeat the fluid removal/flooding procedure for a total of 6 times.
4. Flip out the fluids as described.
5. Invert the plate on absorbent paper to blot off excess fluids.
6. Turn plate right-side-up
7. Add 100 μL TMB Color Developer/well.
8. Incubate 5 minutes.
9. Add 100 μL Color Stopper/well
10. Measure the absorbance at 450 nm against an air blank in a plate reader.

Note: 0 mg/dL should give the highest absorbance on the plate, typically > 1.0. It is a qualitative measure of assay performance.

Data Reduction Nephtrat shows an inverse semi-logarithmic relationship between rat albumin concentration and absorbance at 450 nm. Under normal conditions, this relationship includes the concentrations 0.156-10 mg rat albumin/dL, but variations in laboratory temperature, incubation period and plate handling may cause departures from linearity at the extremes of the standard curve.

Data reduction begins with the determination of mean values for duplicate wells for standard and sample wells. The dose response data should then be plotted as log concentration versus (linear) absorbance. Concentrations that are outside of the linear portion of the curve, if they occur, will do so at the lowest or at the highest concentrations. These value(s) should be dropped from further analysis. The values in the linear range constitute the usable portion of the standard curve and can be subjected to regression analysis. Linear regression of log concentration

vs (linear) absorbance may now be completed. Absorbances for the samples may be substituted in the regression equation to determine raw values for urinary albumin. Values that fall outside of the standard curve cannot be accurately determined, and must be reported as less than the lowest concentration or greater than the highest concentration. Accurate determination may be made on these samples by re-running the assay on the sample after adjusting the dilution procedure. The raw values should be in mg/dL albumin (i. e. anti-log of the predicted value).

Convert the raw values to actual or corrected values by multiplying by the reciprocal of dilution. Thus, if the Basic Procedure is used, the Raw Values each would be multiplied by 10.

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 obtained, processed and stored as discussed above. Rat urine is often contaminated by food and fecal material, and these contaminants present potential sources of error. Therefore, centrifugation of samples is recommended.

Dilute Standard and Samples carefully. For the standards, a single tip may be used to prepare the dilution series. For the experimental samples, a fresh tip should be used for each urine specimen. The tip should be used dry (not prewetted by sample) and washed out in the NHEBSA Diluent by repeated aspiration and expellation.

Limitations:

1. 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.
2. Gross microbiological contamination may affect assay results.
3. Bloody urine specimens are unsuitable for use, even if clarified by centrifugation, since blood is a sign of contamination and since albumin concentrations in the blood are approximately 2000 times those normally found in urine. Semen contains significant levels of albumin and is also a potential source of contamination.

TROUBLE SHOOTING

1. No color appears after adding Color Developer: One or more reagents may have been adversely affected by storage above 8 degrees celsius. 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 incubation to 1 hour.

3. Color in wells is too dark: Repeat the assay and reduce the primary incubation to 15 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. Poor agreement between duplicate wells: This is almost always due to pipetting error. Repeat the assay. Microplate ELISAs are 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.
5. Standard Curve is erratic: This indicates difficulty in serially diluting the standard. Practice serial dilution of standards in the plate. Take care to avoid introducing bubbles and/or foaming during transfer and mixing operations. Avoid scraping the wells during transfer and mixing operations. Alternatively, prepare standard dilutions (and/or sample dilutions) "off-line," e.g. in microfuge tubes, and transfer 100 μ L aliquots to the plate as required.

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6.9.22 Revision

PRODUCT INFORMATION

Cat. #	Description
NR002	Nephrat II ELISA

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