NEPHRAT

For the Quantitation of Rat Urinary Albumin

Nephrat Flow Chart

Fill Wells with Diluent

Add Standard/Serially Dilute

Add Samples

Add Conjugate

Incubate 30 Minutes

Wash

Add Color Developer

Incubate 5 Minutes

Add Color Stopper

Read at 450 nm

Less Than one hour total time

Exocell
1880 JFK Blvd
Suite 200
Philadelphia, PA 19103
www.exocell.com
Phone: (215)557-8021
Fax: (215)557-8053
email:exocellinc@aol.com

Nephrat: For the measurement of rat urine albumin.

Intended Use: Nephrat is an enzyme linked immunosorbent assay (ELISA) for the quantitative determination of albumin in rat urine. It is for research purposes, and is not intended for *in vitro* diagnostic use.

Technical Background: Nephrat is used as a research tool for assessing kidney function in rats. It is simple to perform and highly specific for rat albumin. It is a conventional direct competitive ELISA that recognizes antigen (albumin) in test samples.

To complete the assay, sample and anti-rat albumin antibody-HRP conjugate are added to an albumin-coated well. The antibody binds to the albumin immobilized onto the stationary phase or to the albumin in the fluid phase, hence the notion of competitive binding. After washing, only the antibody-conjugate that has bound to the albumin of the stationary phase will remain in the well, and this is detected using a chromogenic reaction. Color intensity in Nephrat is inversely proportional to the logarithm of albumin 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°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: Your Nephrat kit should contain the following items:

a. 2 Assay Plates
b. 2 NHEBSA Diluent
c. 1 Rat Serum Albumin (RSA) Standard
d. 2 Nephrat Conjugate.
e. 2 TMB Color Developer
f. 2 Acid Color Stopper
g. Instructions

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

Nephrat Assay 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 may be used, 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 is recommended.

Micropipettors capable of delivering 10 and 100 µL are required. A multi-channel pipettors capable of delivering 100 µL is recommended.

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 Nephrat: 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 Nephrat Assay Plate.

The **Basic Procedure** will allow the quantitation of urinary albumin over the range 1.5-100 mg/dL in 80 samples run in duplicate. It requires 20 µL of urine/sample to complete the analysis in duplicate.

The **Alternate Procedure** will allow determination of urinary albumin over a range of 0.3-2,560 mg/dL for 20 samples run at 8 dilutions. Other dilutions may be used to monitor extremely high urinary albumin levels or to measure albumin concentration in rat serum.

Allow reagents and samples to come to room temperature before running the assay. Use only kit reagents and carefully follow instructions. If dilutions are to be made in tubes, use Nephrat Diluent, and follow the Basic Procedure, but omit step 2 and use 100 µL in place of 10 µL in step 3.

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 Nephrat Assay Plate.
2. Transfer 100 µL of RSA each to wells A1 and A2.
3. Mix the fluids thoroughly by repeated pipeting.
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 positons.
4. Tranfer 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 Nephrat 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 Developent 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 absorbant 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 Nephrat 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 adversly affected by storage above 8°C. 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 µL aliquots to the plate as required.

ver. 3.2 17 October 2008