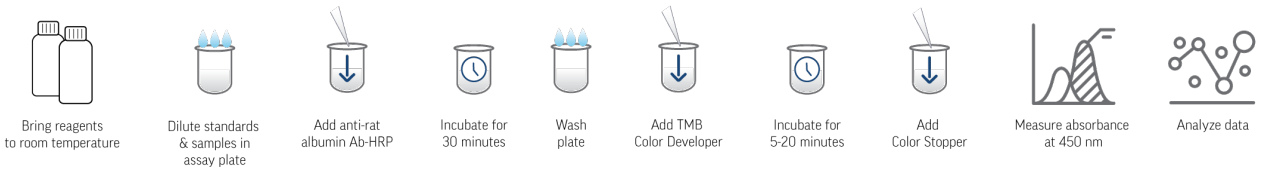
NEPHRAT II ELISA



DESCRIPTION

Intended Use

Nephrat II is an enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of albumin in rat urine.

Technical Background

Nephrat II is an ELISA for assessing the amount of albumin in rat urine, which can be adapted to measure other biological samples from rat. It is a competitive antibody capture ELISA completed in a direct mode (see Appendix, Fig. 1). The anti-rat albumin antibody is conjugated to horseradish peroxidase (HRP), i.e., directly labeled.

To complete the assay, sample and anti-rat Albumin Ab-HRP are added to rat albumin-coated well of a microtiter plate. The antibody either binds with the albumin immobilized to the stationary phase (plate) or with that in the fluid phase, hence the notion of competitive binding. Washing removes anti-rat Albumin Ab-HRP bound to albumin in the fluid phase. Only the anti-rat Albumin Ab-HRP 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 rat albumin concentration in the fluid phase, which means the samples with high amounts of albumin will have light color and samples with low amounts of albumin will have more color. The concentration of rat albumin in an unknown sample can be extrapolated based on the standard curve.

SPECIMEN COLLECTION AND STORAGE

1. Collect samples without preservative.
2. Clarify samples by centrifugation if necessary.
3. Store clarified urine at 4˚C for up to 1 week or at -80˚C for up to 2 months.

MATERIALS NEEDED

KIT CONTENTS

- 2 x 96-well Nephrat II Assay Plates
- 2 x 12 mL NHEBSA (Diluent)
- 1 x 1.8 mL Rat Serum Albumin (RSA) Standard (20 mg/dL)
- 2 x 12 mL anti-rat Albumin Ab-HRP Conjugate
- 2 x 12 mL TMB Color Developer
- 2 x 12 mL Acid Color Stopper

Note: RSA Standard, NHEBSA, and anti-rat Albumin Ab-HRP Conjugate preparations contain 0.05% ProClin® 300 (active components isothiazolones) as preservative. TMB Color Developer is formulated with methanol, dimethylsulfoxide, acetone, 3-3', 5-5' tetramethylbenzidine, and hydrogen peroxide. Acid Color Stopper contains dilute sulfuric acid. These reagents present possible contact hazards, and routine laboratory safety procedures should be followed.

Nephrat II Assay Plates are pre-coated and supplied dry. The plate is used without pretreatment. The controls, standards, samples, and anti-rat Albumin Ab-HRP Conjugate are added directly to the plate.

All kit reagents are supplied in ready-to-use liquid form. Plate washes can be performed using an automatic plate washer or manually. A plate wash procedure using tap water has been found to be suitable in experimental and quality control contexts. However, where tap water is unavailable or unsuitable, we recommend using the Ethos Dilurex™ TEA Wash Buffer (pH 6.8) available at 10x and 1x concentrations.

Micropipettors capable of delivering 10, 50, 100, 120, and 200 µL are required. Multi-channel pipettors capable of delivering 50 and 100 µL are recommended. Finally, a microplate reader equipped to determine absorbance at 450 nm and 650 nm is required.

ASSAY PROCEDURE

Sample Preparation

1. Allow reagents and samples to come to room temperature before running the assay. Place kit components on the laboratory bench for an hour before beginning assay to ensure temperature is fully equilibrated.
2. Do not apply heat to thaw samples.
3. Rat urine can have food or fecal matter contaminants. If so, centrifugation is recommended.
4. For previously frozen samples:
   a. Vortex gently after fully thawed.
   b. Leave samples at room temperature for 1 hour to allow cryoprecipitates to settle out of the fluid column.

Addition of Controls and RSA Standard Serial Dilutions to the Assay Plate

This procedure describes the preparation of controls and seven (7) two-fold dilutions of the RSA standard, which are done in columns 1 and 2 of the 96-well Nephrat II Assay Plate. The remaining columns (3-12) are available for experimental rat urine samples. To design your plate, a fillable plate map is available on the Nephrat II product page or in the resources section of EthosBiosciences.com.

1. Use indelible marker to label 96-well Nephrat II Assay Plate column strips 1-12. This will allow reconstruction of the plate should the strips fall out during the wash procedure.
2. Use a multi-channel pipettor to dispense 100 µL of NHEBSA to wells A1–H2 (see Table 1).
3. Use a single channel pipettor to dispense another 100 µL of NHEBSA to well A1 only. This is a negative control and will be used to "blank" the plate reader.
4. Using a new tip on a single channel pipettor, add 100 µL of RSA Standard (do NOT pre-wet tip) to well B1 and B2. Mix contents thoroughly by gently aspirating and expelling the contents of the well 5 times without introducing bubbles.
5. Place 2 fresh tips onto the multi-channel pipettor.
6. Transfer 100 µL of wells B1 and B2 to well C1 and C2. Mix as before.
7. Continue this procedure through well H1 and H2. Mixing thoroughly.
8. Remove and discard 100 µL from well H1 and H2.
9. Columns 1 and 2 now contain the controls and standards.
10. Continue to the section entitled, *Addition of Experimental Samples to Assay Plate*, either Part A or B.

Table 1. Column 1 and 2 of 96-well assay plate contents.

	COLUMN 1	COLUMN 2
A	"Blank" Final volume = 200 µL NHEBSA	"Positive Control" Final volume = 100 µL NHEBSA
B	10.0 mg/dL RSA 100 µL NHEBSA + 100 µL RSA Std Final volume = 100 µL	10.0 mg/dL RSA 100 µL NHEBSA + 100 µL RSA Standard Final volume = 100 µL
C	5.0 mg/dL RSA 100 µL NHEBSA + 100 µL Well B1 Final volume = 100 µL	5.0 mg/dL RSA 100 µL NHEBSA + 100 µL Well B2 Final volume = 100 µL
D	2.5 mg/dL RSA 100 µL NHEBSA + 100 µL Well C1 Final volume = 100 µL	2.5 mg/dL RSA 100 µL NHEBSA + 100 µL Well C1 Final volume = 100 µL
E	1.25 mg/dL RSA 100 µL NHEBSA + 100 µL Well D1 Final volume = 100 µL	1.25 mg/dL RSA 100 µL NHEBSA + 100 µL Well D2 Final volume = 100 µL
F	0.625 mg/dL RSA 100 µL NHEBSA + 100 µL Well E1 Final volume = 100 µL	0.625 mg/dL RSA 100 µL NHEBSA + 100 µL Well E2 Final volume = 100 µL
G	0.313 mg/dL RSA 100 µL NHEBSA + 100 µL Well F1 Final volume = 100 µL	0.313 mg/dL RSA 100 µL NHEBSA + 100 µL Well F2 Final volume = 100 µL
H	0.156 mg/dL RSA 100 µL NHEBSA + 100 µL Well G1 Final volume = 100 µL	0.156 mg/dL RSA 100 µL NHEBSA + 100 µL Well G2 Final volume = 100 µL

Addition of Experimental Samples to Assay Plate

Accurate determination of rat urinary albumin concentrations depends upon proper sample dilution. In most cases, a 1:10 dilution is sufficient, but collection time and/or kidney function (or dysfunction) may lead to exceptionally high or low albumin concentrations. For initial studies, consider establishing a dilution series to establish the best dilution for subsequent analyses. Part A describes a procedure for creating a dilution series of experimental rat urine samples and accommodates only 10 samples. Part B provides the procedure for using the same dilution of each experimental sample and can evaluate up to 40 samples. Please complete Part A to determine the best dilution for your experiment. Part B is intended for use once the best dilution is established.

A: Assay Plate design for determining best dilution for experimental rat urine samples
This plate design includes the RSA standards in columns 1–2 and serial dilutions of rat urines in columns 3–12. [Table 2 shows only columns 3 and 4, but the remaining columns will have the same dilution series, just different starting samples. This method will evaluate up to 10 different samples.]

1. Set a multi-channel pipettor to dispense 100 µL.
2. Affix 8 fresh tips to the multi-channel pipettor.
3. Pre-wet the tips in NHEBSA.
4. Transfer 100 µL NHEBSA to the balance of the plate, wells A3 through H12.
5. Using a single channel pipettor with fresh tip (do not pre-wet the tip), add 100 µL of the first urine sample to well A3. Mix contents thoroughly by gently aspirating and expelling the contents of the well 5 times without introducing bubbles.
6. Repeat adding 100 µL of the rat urine samples into the remaining wells of row A (A4–A12), using a new tip for each.
7. Place 10 fresh tips onto the multi-channel pipettor.
8. Transfer 100 µL of well A3 through A12 into the wells one row down. Mix as before.
9. Continue this procedure through row H. Mixing thoroughly.
10. Remove and discard 100 µL from row H.

Table 2: Plate design to establish best dilution for experimental rat urine samples.

	1	2	3	4
A	200 µL NHEBSA	100 µL NHEBSA	100 µL NHEBSA + 100 µL urine sample 1	100 µL NHEBSA + 100 µL urine sample 2
B	100 µL 10.0 mg/dL RSA	100 µL 10.0 mg/dL RSA	100 µL NHEBSA + 100 µL of A3	100 µL NHEBSA + 100 µL of A4
C	100 µL 5.0 mg/dL RSA	100 µL 5.0 mg/dL RSA	100 µL NHEBSA + 100 µL of B3	100 µL NHEBSA + 100 µL of B4
D	100 µL 2.5 mg/dL RSA	100 µL 2.5 mg/dL RSA	100 µL NHEBSA + 100 µL of C3	100 µL NHEBSA + 100 µL of C4
E	100 µL 1.25 mg/dL RSA	100 µL 1.25 mg/dL RSA	100 µL NHEBSA + 100 µL of D3	100 µL NHEBSA + 100 µL of D4
F	100 µL 0.625 mg/dL RSA	100 µL 0.625 mg/dL RSA	100 µL NHEBSA + 100 µL of E3	100 µL NHEBSA + 100 µL of E4
G	100 µL 0.313 mg/dL RSA	100 µL 0.313 mg/dL RSA	100 µL NHEBSA + 100 µL of F3	100 µL NHEBSA + 100 µL of F4
H	100 µL 0.156 mg/dL RSA	100 µL 0.156 mg/dL RSA	100 µL NHEBSA + 100 µL of G3	100 µL NHEBSA + 100 µL of G4

[**Note:** Every well, except well A1, in the 96-well Nephrat II Assay Plate should have 100 µL of liquid.]

B. Assay Plate design for testing rat urine samples with the same dilution
The following provides an example of how to analyze rat urine samples using a 1:10 dilution. Wells A3 & A4 through H11 & H12 will contain rat urine samples diluted 1:10 in duplicate wells for a total of 40 different samples in this design. You may need to adjust the dilution based on results obtained in *Section A, Assay Plate design for establishing best dilution for experimental rat urine samples.*

1. Set a multi-channel pipettor to dispense 90 µL.
2. Add 8 fresh tips.
3. Pre-wet the tips in NHEBSA.
4. Transfer 90 µL NHEBSA to the balance of the plate, wells A3 through H12.
5. Use a fresh tip to add 10 µL rat urine sample #1 to well A3. Repeat in well A4.
6. Use a fresh tip to add 10 µL of the next sample to well B3. Repeat in well B4.
7. Continue in this manner taking care to use a fresh tip for each sample.

Table 3: Plate design for 40 experimental rat urine samples diluted 1:10.

	1	2	3	4
A	200 µL NHEBSA	100 µL NHEBSA	90 µL NHEBSA + 10 µL sample U1	90 µL NHEBSA + 10 µL sample U1
B	100 µL 10.0 mg/dL RSA	100 µL 10.0 mg/dL RSA	90 µL NHEBSA + 10 µL sample U2	90 µL NHEBSA + 10 µL sample U2
C	100 µL 5.0 mg/dL RSA	100 µL 5.0 mg/dL RSA	90 µL NHEBSA + 10 µL sample U3	90 µL NHEBSA + 10 µL sample U3
D	100 µL 2.5 mg/dL RSA	100 µL 2.5 mg/dL RSA	90 µL NHEBSA + 10 µL sample U4	90 µL NHEBSA + 10 µL sample U4
E	100 µL 1.25 mg/dL RSA	100 µL 1.25 mg/dL RSA	90 µL NHEBSA + 10 µL sample U5	0 µL NHEBSA + 10 µL sample U5
F	100 µL 0.625 mg/dL RSA	100 µL 0.625 mg/dL RSA	90 µL NHEBSA + 10 µL sample U6	90 µL NHEBSA + 10 µL sample U6
G	100 µL 0.313 mg/dL RSA	100 µL 0.313 mg/dL RSA	90 µL NHEBSA + 10 µL sample U7	90 µL NHEBSA + 10 µL sample U7
H	100 µL 0.156 mg/dL RSA	100 µL 0.156 mg/dL RSA	90 µL NHEBSA + 10 µL sample U8	90 µL NHEBSA + 10 µL sample U8

[**Note:** Every well, except well A1, in the 96-well Nephrat II Assay Plate should have 100 µL of liquid.]

Primary Incubation

At this point, every well, except well A1, in the 96-well Nephrat II Assay Plate should have 100 µL of liquid. This section describes the procedure for the reaction with anti-rat Albumin Ab-HRP.

1. Skip well A1. This is the negative control.
2. Pour contents of the anti-rat Albumin Ab-HRP into a reservoir.
3. Affix 7 tips to a multi-channel pipettor.
4. Pre-wet the tips with anti-rat Albumin Ab-HRP and draw up 100 µL.
5. Dispense the 100 µL volume into column 1 (B1-H1).
6. Add an eighth tip, pre-wet as before.
7. Starting with well A2, add 100 µL of anti-rat Albumin Ab-HRP to the remain wells.
8. Cover and incubate the plate for 30 mins at room temperature.

[**Note:** To ensure that the plate is kept in a stable environment, this incubation should be done in a closed container with a moisture saturated atmosphere. To create the correct conditions, plastic container with a lid can be lined with a water moistened paper towel.]

[**Note:** Each well should have a total of 200 µL of liquid.]

Plate Washing Procedure

1. Wash plate using an automatic plate washer a total of 10 times.
2. Alternatively, plates can be washed manually using the following:
   a. Remove fluids from the wells by aspiration or by flipping plate over a sink.
   b. Fill wells to overflowing with water or wash buffer.
   c. Remove fluids as before.
   d. Repeat for a total of 10 washes.
3. Invert the plate on a paper towel, tapping gently to remove any excess fluid.

Color Development and Measurement

1. Add 100 µL of TMB Color Developer to each well with a multi-channel pipettor. Pre-wet tips as above.
2. Incubate 5–20 mins in a closed container with a moisture saturated atmosphere to allow color to develop.
3. Add 100 µL of Color Stopper to each well using a multi-channel pipettor. Pre-wet tips as above.
4. Examine the plate. The negative control in well A1 should have little to no color. The positive control in well A2 should be the most intensely colored well on the plate. The rest of the wells should show color intensity in between these two extremes.
5. Set plate reader to measure the optical density at 450 nm first, using well A1 as a blank.
6. Measure the optical density again, but at 650 nm to subtract for any plate imperfections such as scratches.

ANALYSIS

The log of the RSA concentration and OD 450 will show an inverse relationship. Under most laboratory conditions, each of the standard dilutions should fit the model. Variations in laboratory temperatures can place some RSA standards out-of-range.

If using modeling software, this kit has enough dilutions to use the 5PL non-linear regression model. If the dose-response is symmetrical enough, the model will collapse into a 4PL model. If no modeling software is available, the following procedure can be used in MS Excel®.

1. Prepare a spreadsheet entering appropriate data including standard concentration, sample dilution, and absorbance data. Determine the mean for replicate wells.
2. Prepare a semi-logarithmic plot of standard dilutions with the log10 [RSA] on the x-axis and mean absorbance at 450 nm on the y-axis. This is the standard curve.
3. The data that fall into the linear portion of the standard curve constitute the usable portion of the assay.
4. Subject these data to semi-logarithmic analysis to yield a mathematical model, of the form: log10[RSA] = m A450 + b Where m is the slope and b is the y-intercept.
5. RSA concentration is determined by taking the anti-log of the calculated values from this equation.
6. Multiply by the inverse dilution factor to determine the undilute concentration of rat albumin in each sample.

QUALITY CONTROL

Record Keeping: It is good laboratory practice to record the lot numbers and dates for the kit components and reagents used for each assay.

LIMITATIONS

- Samples must not contain inhibitors of HRP, i.e. sodium azide. These will affect results.
- 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.
- Bloody urine specimens are unsuitable for use, even if clarified by centrifugation, since blood flow 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.

TROUBLESHOOTING

1. No color appears after adding Color Developer: One or more reagents may have been adversely 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 with anti-rat Albumin Ab-HRP conjugate to 1 hour.
3. Color in wells is too dark: Repeat the assay and reduce the initial incubation with anti-rat Albumin Ab-HRP to 15 minutes.
4. If color is dark and the standard dilutions fail to show the appropriate concentration-dependent-response, Color Developer may have been contaminated with anti-rat Albumin Ab-HRP or the plate was poorly washed. Repeat the assay and take care in the pipetting and in the washing operations.
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.
7. 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) in microfuge tubes, and transfer 100 µL aliquots to the plate as required.

CREATININE COMPANION ASSAY

The Creatinine Assay is designed for companion use with Exocell's albumin ELISAs, allowing expression of results as µg protein (i.e. albumin) per mg creatinine in the urine. This is typically used as a normalized value to compare samples.

PRODUCT INFORMATION

CAT. #	DESCRIPTION
NR002	Nephrat II ELISA
1012	Creatinine Companion (Creatinine Assay)

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TRADEMARKS

APPENDIX

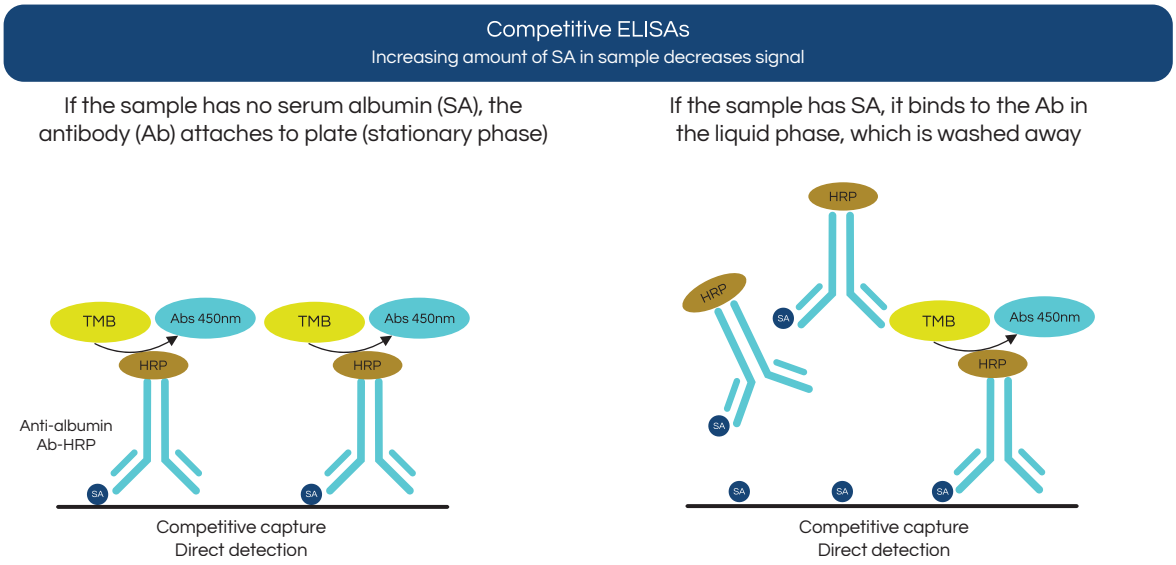

Fig. 1. Competitive ELISAs have decreasing signals when albumin is present in samples. The HRP molecule is directly attached to the Fc region of the anti-albumin antibody, thus eliminating the need for adding a labeled secondary antibody. The left diagram represents what happens in well A2 of this kit. The right diagram represents the addition of SA standards in wells B1 and B2 through H1 and H2. The more albumin in the sample, the lower the absorbance reading since the Anti-Albumin Ab-HRP conjugate will wash away in the liquid phase rather than binding to the plate.