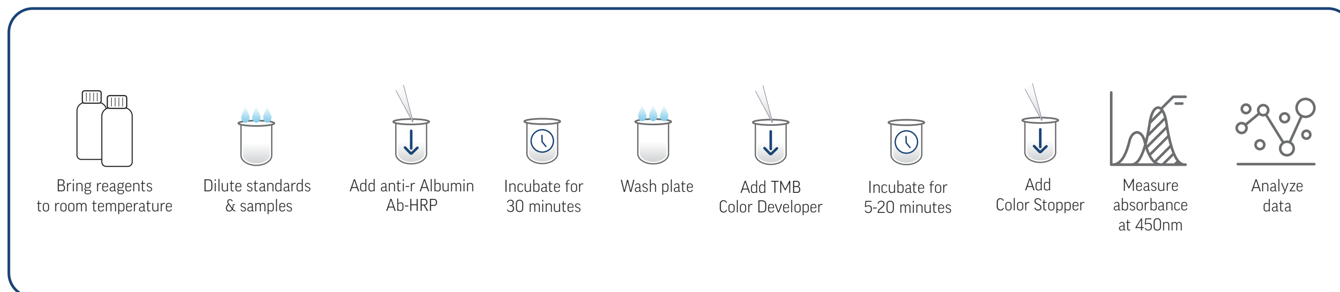
Ethos Biosciences

NEPHRAT II ELISA



DESCRIPTION

Nephrat II is an Enzyme Linked Immunosorbent Assay (ELISA) for the quantitative measurement of rat albumin concentration in biological samples.

Intended Use

Nephrat II is designed for use with urine specimens but is readily adapted for use with other biological samples. The assay is for research applications, and is NOT intended for diagnostic use.

Technical Background

Nephrat II is a direct antibody capture competitive assay. The Nephrat II assay plate is coated with rat albumin (r albumin); this is the stationary or sorbent phase antigen. Samples (containing r albumin) are added to the plate, these samples present the antigen in a mobile phase. Then, an anti-r albumin antibody-HRP conjugate is added to the plate.

The antibody moiety of the conjugate binds to rat albumin of the stationary phase or to that in the mobile phase, hence the notion of competitive binding. The reactants and immunocomplexes that remain in the mobile phase are then washed away, and only the conjugate bound to the sorbent phase antigen remains.

Bound conjugate is determined using TMB (3,3',5,5'-tetramethylbenzidine), a chromogenic substrate for HRP. It is added to the plate and the color is allowed to develop for 5-20 minutes. The development is stopped with the addition of dilute acid.

The assay is standardized by running serial dilutions of a standard along with the samples. The optical densities of the wells containing diluted standard and those containing samples are determined using a plate reader set to measure absorbance at 450 nm.

The color intensity is inversely proportional to the log of r albumin concentration of the mobile phase. Therefore, (mobile phase) samples with high concentrations of r albumin will produce lightly colored wells, whereas those with little or no r albumin will be intensely colored. Quantitative results are obtained by modeling the dose-response measured for the standards and calculating the unknown sample concentrations from measured absorbances.

SPECIMEN COLLECTION AND STORAGE

Collect samples without preservative and clarify them by centrifugation if necessary. Store clarified urine at 4ºC for up to 1 week or at -60ºC 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. 2 x 96-well Nephrat II Assay Plates
2. 1 x 1.8 mL Rat Serum Albumin (RSA) Standard
3. 2 x 12 mL NHEBSA (Diluent)
4. 2 x 12 mL Anti-RSA Ab-HRP
5. 2 x 12 mL TMB Developer
6. 2 x 12 mL Acid Stopper

NHEBSA, RSA Standard and HRP Conjugate are formulated in buffers that contain preservative (isothiazalones). TMB Color Developer is formulated with methanol, dimethylsulfoxide, acetone, 3-3', 5-5' tetramethylbenzidine, and hydrogen peroxide. The color stopper contains dilute sulfuric acid. These reagents present possible contact hazards, and routine laboratory safety procedures should be followed.

The kit components are ready to use, but should be allowed to come to room temperature before beginning the assay. This is easily done by placing the kit components on the laboratory bench for an hour before beginning. The assay plate is supplied dry, but is used in the assay without pretreatment. The controls, standards, samples and HRP conjugate are added directly to the plate.

The assay calls for addition of control, standard and sample to the plate. The assay volume is 100 µL. These additions should be done using an appropriate pipettor, i.e. one adjustable to 100 µL. For sample dilution, 10 µL aliquots of sample are added to 90 µL of diluent; this requires pipettors that may be set to deliver these volumes. The HRP-Conjugate is added at 100 µL per well. This is best done using a multi-channel pipettor.

The plate is incubated for 30 minutes after addition of the HRP-Conjugate. To insure that the plate is kept in a stable environment; this incubation should be done in a closed container in moisture saturated atmosphere. A Tupperware plastic container with a water moistened paper towel (placed on the bottom of the tray) has proven to be a satisfactory incubation chamber.

After 30 minutes, unreacted reagents and rAlbumin-anti-rAlbumin PAb-HRP conjugate complexes in the solution phase are removed by washing the plate. For most laboratories, cold tap water is acceptable as a wash buffer. Where there is limited access to a sink, the assay plate can be washed with wash buffer. Ethos' TEA Wash Buffer is recommended.

The final reaction of the assay is using HRP substrate TMB. This is added at 100 µL/well. After a suitable development period, the reaction is stopped using dilute sulfuric acid. Both of these additions should be completed using a multi-channel pipettor.

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	1	2	3	4	5	6	7	8	9	10	11	12
A	C0	C1	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	10.000	10.000	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	5.000	5.000	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	2.500	2.500	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	1.250	1.250	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	0.625	0.625	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	0.313	0.313	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	0.156	0.156	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

To secure data; the plate must be "read" using a plate reader equipped to measure optical density at 450 nm. The resulting data array should be analyzed using appropriate software.

ASSAY PROCEDURE

Please read the entire insert fully before beginning. This will allow preparation of materials and equipment that will facilitate assay completion. Nephrat II dilutions of both standard, and samples are completely directed in the plate.

The plate design includes controls, and duplicate wells of serially diluted standard and rat urines.

1. Use indelible marker to label strips 1-12
2. The plate design includes controls, and duplicate wells of serially diluted standard and rat urines
3. Using a multi-channel pipettor, dispense 100 µL of NHEBSA diluent into wells A1-H2
4. Reset the pipettor, and dispense 90 µL NHEBSA Diluent into wells A3-H12
5. With a single-channel pipettor, add 100 µL NHEBSA to well A1. This is the C0 or Negative control, and will be used to "blank" the plate reader
6. With a single-channel pipettor; transfer 100 µL of RSA standard into wells B1 and B2
7. Equip multi-channel with two tips, and set to deliver 100 µL
8. Mix the contents of B1, B2 by aspirating and expelling the fluids 5 x
9. Transfer 100 µL from these wells to wells C1, C2
10. Mix the contents as before
11. Transfer 100 µL from C1, C2 to wells D1, D2
12. Mix as before
13. Continue the dilution as described through to wells H1,2
14. Remove 100 uL from H1, H2 and discard
15. Well A1 has 200 µL NHEBSA
16. Well A2 has 100 µL NHEBSA
17. Wells B1-H2 have serial diluted RSA standard in duplicate wells from 10 mg/dL (wells B1, B2)-0.156 mg/dL (wells H1, H2)
18. Use a fresh tip and add 10 µL rat urine to each of wells A3, A4
19. Use a fresh tip and add 10 µL of the next specimen to wells B3, B4
20. Continue in this manner taking care to use a fresh tip for each specimen
21. Wells A3, A4 – H11, H12 have rat urine diluted 1:10 in duplicate wells, 100 µL/well

Add anti-rAlbumin-HRP Conjugate:

1. Pour contents of anti-rAlbumin-HRP conjugate into a reservoir Adjust the multi-channel pipettor to deliver 100 µL
2. Equip it with 7 tips
3. Pre-wet the tips in HRP-Conjugate Dispense HRP Conjugate into wells B1-H1
4. Add an eighth tip, and pre-wet the tips again in HRP-Conjugate Dispense 100 µL volumes of HRP-Conjugate into wells A2-H12 All the wells should have 200 µL in them
5. Incubate for 30 minutes at room temperature

Wash the plate:

1. Set the cold water tap to a flow rate that might be used to fill a water glass (flow rate is not critical)
2. Flip the plate out into the sink
3. Hold the plate by the long edges, and tilted down slightly (away from the operator)
4. Pass each well under the flowing water until all of the wells a filled
5. Flip out into the sink as before
6. This is one wash cycle
7. Continue this until the plate is washed through 10 wash cycles
8. After flipping the water out the last time, invert the plate on a paper towel to blot off excess fluid

Develop the plate:

1. Pour the TMB Color Developer (Developer) into a clean/fresh reservoir
2. Set the multi-channel pipettor to deliver 100 µL
3. Affix 8 fresh tips
4. Pre-wet the tips in Developer
5. Dispense Developer into all of the wells, 100 µL/well
6. Place plate back into the incubation chamber, and cover
7. Develop for 5-20 minutes
8. Pour Acid Stopper into a fresh reservoir
9. Affix 8 fresh tips to the multi-channel pipettor
10. Be sure it is set to deliver 100 µL
11. Dispense Acid Stopper into the plate at 100 µL/well

Read the plate:

1. Turn on plate reader
2. If necessary, use the plate map to set up a template in the machine software
3. Set controls to read optical density at 450 nm

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Nephrat II: Plate Map: Method 2:

	1	2	3	4	5	6	7	8	9	10	11	12
A	C0	C1	U1 1:2	U2 1:2	U3 1:2	U4 1:2	U5 1:2	U6 1:2	U7 1:2	U8 1:2	U9 1:2	U10 1:2
B	10.000	10.000	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
C	5.000	5.000	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8
D	2.500	2.500	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16
E	1.250	1.250	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32
F	0.625	0.625	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64
G	0.313	0.313	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128
H	0.156	0.156	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256

4. Choose 650 nm as second wavelength
5. The OD at 650 is subtracted from the OD at 450 to correct for scratches
6. Set the machine to use well A1 (this is negative control) as "blank"
7. Install the plate
8. "Read" the plate
9. Well A2 is the positive control, and should have the highest OD on the plate

Assay Method 2: Refer to Second Plate Map

1. Use an indelible marker to label the strips.
2. Pour NHEBSA into a fresh reservoir.
3. Set a multi-channel pipettor to dispense 100 µL.
4. Affix 8 fresh tips
5. Pre-wet the tips in NHEBSA
6. Transfer 100 µL to every well of the plate
7. Use a single-channel pipettor to add 100 µL of NHEBSA to well A1. This will (again) be C0 or the negative control
8. Using a single channel pipettor transfer 100 µL volumes of standard into wells B1, B2
9. Using the single-channel pipettor with a fresh tip for every urine specimen; transfer 100 µL of rat urine to well A3.
10. Continue adding urine from each specimen into wells A4-A12.
11. Use the multi-channel pipettor to serially dilute the standard in the plate as described before.
12. Use fresh tips, mix the contents of wells A3-12 by aspirating and expelling the fluid 5 times.
13. Serially dilute the samples in the same way that the standards were done
14. Take care to remove 100 µL from the final wells (H1-12)

Refer to procedure one to complete processing for this method.

Data Reduction

Nephrat II data show an inverse relationship between log [rat albumin] and OD 450. Under normal laboratory conditions, this relationship will include all concentrations in the standard curve, 0.156-10 mg/dl, but variations in laboratory temperature may place some results out-of-range.

Data reduction should begin with determining mean absorbances for both standard dilutions and sample dilutions. The rat albumin standards should be plotted on a graph relating log [concentration] to OD. All values that fall in a line for the standard curve may then be modeled using a semi-logarithmic method. However; the data may be better analyzed using a non-linear regression model.

Nephrat II data are well fit using a 4PL or 5PL model, and a curve-fitting program will allow ready calculation of unknown samples. Multiplying the estimated concentrations by reciprocal dilution will return undilute rat albumin concentrations for the specimens.

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.

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.

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.

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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.
4. 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.
5. Poor agreement between duplicate wells: This is almost always due to pipetting error. Repeat the assay.
6. 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.
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) "off-line," e.g. in microfuge tubes, and transfer 100 µL aliquots to the plate as required.

PRODUCT INFORMATION

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

Order today at **EthosBiosciences.com**

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