Albuwell Hu
Assay Flow Chart

Bring Reagents to Room Temperature

Dilute Standards and Samples

Add Aliquots to Wells

Add anti hSA-Ab-HRP

Incubate 30 Minutes

Wash Plate

Add TMB Color Developer

Incubate 5-10 Minutes

Add Color Stopper

Measure Absorbance at 450 nm

Analyze Data

Exocell: A Division of
Ethos Biosciences, Inc.
1880 JFK Blvd.
Philadelphia, PA 19103

Phone: (215) 557-8021
Fax: (215) 557-8053
e-mail: exocell@verizon.net

Albuwell Hu: For the quantitative measurement of microalbuminuria in human specimens.

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

Technical Background: Albuwell Hu is intended for use as an in-vitro tool for assessing kidney function in humans. It is simple to perform and highly specific for human 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-human albumin Ab-HRP are added to human 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^{O}C for up to 1 week or at -60^{O}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 Albuwell Hu kit should contain the following items:

1. 2 Albuwell Assay Plates
2. 2 NHEBSA (Diluent)
3. Human Serum Albumin (hSA) Standard
4. 2 Anti-human Albumin Ab-HRP Conjugate
5. 2 Color Developer
6. 2 Color Stopper
7. Instructions

hSA Standard, NHEBSA, Rabbit anti-human Albumin Ab-HRP Conjugate preparations contain 0.05% Proclin 300 (active components isothiazolones) as preservative. Color Stopper contains dilute (2.0 N) sulfuric acid

Albuwell 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 has been shown to be suitable in experimental and quality control contexts, 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 (preservative may be omitted if the buffer is freshly prepared) may be used where tap water is unavailable or proves unsuitable.

Micropipettors capable of delivering 10, 50, 100 and 120 uL are required. Multi-channel pipettors capable of delivering 50 and 100 uL are recommended. In addition, small test tubes are required to complete dilutions (microfuge tubes work well in this application). Finally, a microplate reader equipped to determine absorbance at 450 nm is required.

Assay Procedure: Allow reagents and samples to come to room temperature before running the assay.

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

1. Prepare 8 microfuge tubes with 200 uL of NHEBSA per tube.
2. Label the tubes “C” and 1-7, respectively
3. Transfer 200 uL of hSA Standard to tube 1.
4. Mix contents by aspirating and expelling the fluids 5 times.
5. Transfer 200 uL of solution from tube 1 to tube 2.
6. Mix as before.
7. Continue this procedure through tube 7.
8. Tubes 1-7 now contain dilutions of 10.0, 5.0, 2.5, 1.25, 0.625, 0.313 and 0.156 µg hSA/mL respectively.

Preparation of Urine Sample Dilutions: Accurate determination of urinary albumin depends upon proper sample dilution. In most cases, a 1:21 dilution is sufficient, but collection time and/or kidney function (or dysfunction) may lead to exceptionally high or exceptionally low concentrations. For initial studies it is wise to complete the analysis at three concentrations, i. e. 1:21, 1:42 and 1:84. The results obtained will allow the choice of the best (single) dilution for subsequent analyses.

The following example illustrates a 1:21 dilution protocol.

1. Prepare and label a microfuge tube for each sample.
2. Add 200 uL NHEBSA to to each tube
3. Use a dry fresh tip to transfer 10 uL of sample to the appropriate tube, wash out the tip by repeated aspiration and expellation in the tube.
4. Vortex the tube briefly.
5. Continue this procedure for the rest of the samples.
6. Each sample is now diluted 1:21 in NHEBSA

Addition of Controls, Standard hSA Dilutions and Samples to the plate: Label

the strips with an indelible marker, 1-12. This will allow reconstruction of the plate should strips fall out during the washing procedures. The diluted standards and samples may be added directly to the dry plate.

This plate design includes two controls: a negative control termed C0, and a positive control termed C1. These are placed in wells A1 and A2, respectively. All other wells receive either diluted standard or diluted sample. The assay volume is 50 uL.

1. Add an 100 uL aliquot of NHEBSA from the tube C to well A1. This is the negative control "C0" and will be used to standardize or "blank" the microplate reader.
2. Add 50 uL of NHEBSA from tube C to well A2. This is the positive control "C1," and and serves as a qualitative indicator of assay performance.
3. With a fresh tip, pre-wet the tip in standard dilution number 7, and transfer 50 uL aliqouts to wells H1 and H2.
4. With the same tip, pre-wet/rinse the tip in standard dilution number 6, and transfer 50 uL aliquots to wells G1 and G2.
5. Continue transferring diluted standard to the plate in this fashion, i. e. pre-wetting/rinsing the tip and transferring aliquots of standard in order.
6. Using a new tip, pre-wet the tip in the first diluted sample, and transfer 50 uL aliquots to wells A3 and A4.
7. Taking care to change the tip, and to pre-wet it each time; continue adding diluted samples to the plate.
8. The plate now contains controls and diluted standards in wells A-H, 1,2, and diluted experimental samples in duplicate in the balance of the plate.

Primary Incubation: Reaction with Anti-human Albumin Ab-HRP conjugate

1. Add 50 uL of Anti-human Albumin Ab-HRP conjugate to Wells A2-A12, and B-H 1-12.
2. Cover and incubate the plate for 30 minutes.

Wash Plate: Use a plate washer or wash plates by hand as follows:

1. Remove fluids from the well, ie. aspirate off fluids or flip them out into a sink.
2. Fill wells to over-flowing with water or wash buffer.
3. Remove fluids as before.
4. "2" and "3" constitute a wash cycle.
5. Repeat the process to yield a total of 10 wash cycles.
6. Invert the plate on a paper towel and tap gently to remove excess fluids.

Color Development:

1. Add 100 uL of Color Developer to each well.
2. Develop 5- 10 minutes
3. Add 100 uL of Color Stopper to each well.

Analysis: Examine the plate. The negative control well, C0 which is in well A1, should have little or no color, but the positive control well, C1 which is in well A2, should be the most intensely colored well on the plate. The rest of the wells should show absorbances intermediate between these extremes.

This analysis assumes that computer and analysis software, e.g. MS Excel, is available.

1. Use a plate reader to determine the absorbances at 450 nm, use the C0 well in A1 to “blank” the reader.
2. Prepare a spreadsheet entering appropriate data including standard dilution, concentration, sample dilution and absorbance data. Determine the mean for replicate wells.
3. Prepare a semi-logarithmic plot of standard dilutions with the log [hSA] on the x-axis and mean absorbance on the y axis. This is the dose-response or standard curve.
4. The data that fall into the linear portion of the dose-response curve constitute the usable portion of the assay.
5. Subject these data to semi-logarithmic analysis to yield a mathematical model, of the form

$$\log_{10} [\, hSA \,] = m\, A_{450} + b$$

6. hSA concentration is determined by taking the anti-log of the calculated values from this equation.
7. Multiply by 21 (or inverse dilution factor) to correct for the dilution.

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.

Sample Handling: The samples should be secured, processed and stored as discussed above. Human urine can be contaminated, and these contaminants present potential sources of error.

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.

Limitations:

1. Samples must not contain inhibitors for HRP, i.e. sodium azide. These will affect results
2. 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.
3. Gross microbiological contamination may affect assay results.
4. 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.

Trouble Shooting:

1. No color appears after adding Color Developer: One or more reagents may have been adversely affected by storage above 8^{o}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 incubation with the conjugate to 1 hour.
3. Color in wells is too dark: Decrease the development time. If a 5 minute development is still too dark, repeat the assay and reduce the secondary 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. Color in sample well(s) is darker or lighter than lowest or highest concentrations of the standard curve. Change sample dilution protocol appropriately.
6. Poor agreement between duplicate wells: This is almost always due to pipetting error. Repeat the assay.
7. 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.

Rev 1.1_GTL Nov 2018