Albuwell M: A murine microalbuminuria ELISA.

Intended Use: Albuwell M is an enzyme linked immunosorbent assay (ELISA) for the quantitative determination of albumin in mouse urine.

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

To complete the assay, sample and anti-mouse albumin Ab-HRP are added to mouse albumin-coated well. The antibody interacts and binds with the albumin immobilized onto the stationary phase or with that in the fluid phase, hence the notion of competitive binding. Washing removes unbound Ab-HRP:Albumin and other reactants of the fluid phase from the well. Only the antibody-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 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 Albuwell M kit should contain the following items:
1. 2 Albuwell M Test Plates
2. 2 NHEBSA (Diluent)
3. Mouse Serum Albumin (MSA) Standard
4. 2 Anti-mouse Albumin Ab-HRP Conjugate
5. 2 Color Developer

Add Aliquots to Wells
Incubate 30 minutes
Wash Plate
Add TMB Color Developer
Incubate 5-10 Minutes
Add Acid Color Stopper
Measure Absorbance at 450 nm
Complete Calculations

Assay Flow Chart

Dilute Standards and Samples
Add Standards
Add anti-Mouse Albumin Ab-HRP
Incubate 30 minutes
Wash Plate
Add TMB Color Developer
Incubate 5-10 Minutes
Add Acid Color Stopper
Measure Absorbance at 450 nm
Complete Calculations

Preparation of Urine Sample Dilutions:
Accurate determination of urinary albumin depends upon proper sample dilution. In most cases, a 1:13 dilution is sufficient, but collection methods and animal 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. undilute, 1:20 and 1:80. The results obtained will allow the choice of the best (single) dilution for subsequent analyses.

The following example illustrates a 1:13 dilution protocol.

1. Prepare and label a microfuge tube for each sample.
2. Add 120 µL NHEBSA to each tube.
3. Use a dry fresh tip to transfer 10 µL 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 1:13 in NHEBSA.

Addition of Controls, Standard MSA 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 µL.
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 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 aliquots 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-mouse Albumin Ab-HRP conjugate

1. Add 50 uL of Anti-mouse 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, i.e. 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 [MSA] 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

\[
\log_{10}[\text{MSA}] = m_{450} + b
\]

6. MSA concentration is determined by taking the anti-log of the calculated values from this equation.

7. Multiply by 13 (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. Mouse urine is often contaminated by food and fecal material, and these contaminants present potential sources of error. Centrifugation to clarify 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.

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 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 80°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: 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.

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