K-ASSAY®

KAMIYA BIOMEDICAL COMPANY

Rat Microalbumin ELISA

For the quantitative determination of microalbumin in rat biological samples

Cat. No. KT-354

For research use only.

Rev. 15927354

PRODUCT INFORMATION

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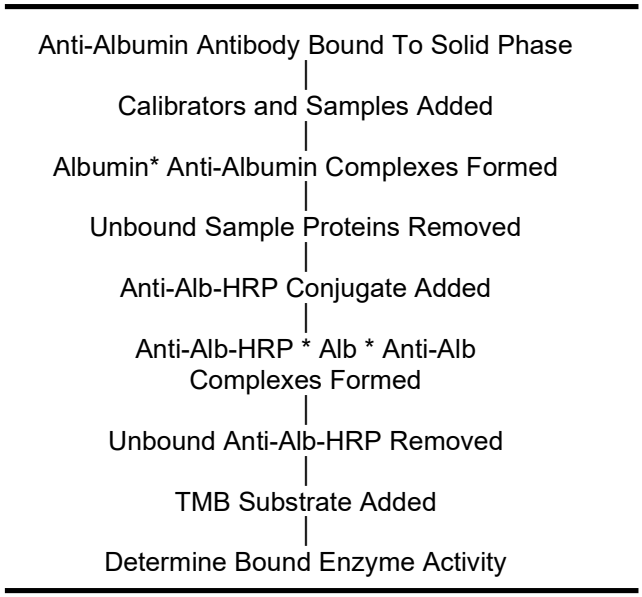
INTENDED USE
The Rat Microalbumin ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of microalbumin in rat biological samples. For research use only.

INTRODUCTION
Albumin (Alb) is an amazing polyfunctional protein contributing to homeostasis through mechanisms of hemodynamics, transport and nutrition. Albumin is found both intra- and extravascularly in all mammals and many lower vertebrates. It is a molecule of about 67,000 daltons, synthesized by the liver. Normally only very trace amounts of albumin escape reabsorption by kidney glomeruli and are excreted into the urine. Many occult diseases can cause kidney damage which may result in excessive amounts of serum proteins, including albumin, to be excreted by the kidney and into the urine. This ELISA can be used to measure albumin in serum, tissue extracts and other biological fluids.

PRINCIPLE
The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the albumin present in the sample reacts with the anti-Alb antibody, which has been absorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-Alb antibody conjugated with horseradish peroxidase (HRP), are added. These HRP-conjugated antibody forms a complex with the previously bound albumin. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of albumin in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of albumin in the test sample. The quantity of albumin in the test sample can be interpolated from the calibration curve constructed from the calibrators and corrected for sample dilution.

Figure 1.



COMPONENTS

1. **Diluent Concentrate**
   One bottle containing 50 mL of a 5X concentrated diluent buffer.
2. **Wash Solution Concentrate**
   One bottle containing 50 mL of a 20X concentrated wash solution.
3. **Enzyme-Antibody Conjugate Concentrate**
   One vial containing 150 µL of a 100X concentrated affinity-purified anti-rat albumin antibody conjugated with HRP in stabilizing buffer.
4. **MB Substrate Solution**
   One bottle containing 12 mL of TMB and hydrogen peroxide in citric acid buffer at pH 3.3.
5. **Stop Solution**
   One bottle containing 12 mL of 0.3 M sulfuric acid.
   WARNING: Avoid contact with skin.
6. **Microtiter Plate**
   Twelve removable eight-well strips in a well holder frame. Wells are coated with affinity-purified anti-rat albumin
7. **Rat Microalbumin Calibrator**
   One vial containing a lyophilized Rat Microalbumin Calibrator.
8. **Positive Control**
   One vial containing 50 µL of serum with 0.1% sodium azide. See the Control Certificate for the concentration.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes (2 µL to 200 µL) for making and dispensing dilutions
- Test tubes
- Squirt bottle or microplate washer/aspirator
- Distilled or de-ionized H_2O
- Microplate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Centrifuge for sample collection
- Anticoagulant for plasma collection

PRECAUTIONS

1. Read the instructions carefully before beginning the assay.
2. This kit is for research use only.
3. Great care has been taken to ensure the quality and reliability of this product. However, it is possible that in certain cases, unusual results may be obtained due to high levels of interfering factors.
4. Preservatives
   - Positive Control contains 0.1% sodium azide.
5. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
6. Other precautions:
   - Do not interchange kit components from different lots.
   - Do not use kit components beyond the expiration date.
   - Protect reagents from direct sunlight.
   - Do not pipette by mouth.
   - Do not eat, drink, smoke, or apply cosmetics where reagents are used.
   - Avoid all contact with the reagents by using gloves.
   - Stop solution contains diluted sulfuric acid. Irritation to the eyes and skin is possible. Flush with water after contact.

STORAGE AND STABILITY

1. **Complete Kit**
   The expiration date for the kit is stated on the outer label. The recommended storage temperature is 4°C. All components should be stable up to the expiration date if stored and used per this kit protocol insert. **Note: See long-term storage recommendations below for the Rat Microalbumin Calibrator and Positive Control**
2. **Diluent Concentrate**
   The 5X Diluent Concentrate is stable until the expiration date of the kit. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4°C.
3. **Wash Solution Concentrate**
   The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4°C.
4. **Enzyme-Antibody Conjugate**
   Undiluted anti-Alb-HRP conjugate should be stored at 4°C in the dark (protect from light) and **diluted immediately prior to use**. The undiluted conjugate solution is stable until the expiration date.
5. **TMB Substrate Solution**
   The TMB Substrate Solution should be stored at 4°C in the dark (protect from light) and is stable until the expiration date.
6. **Stop Solution**
   The Stop Solution should be stored at 4°C and is stable until the expiration date.
7. **Microtiter Plate**
   Anti-rat albumin coated wells are stable until the expiration date of the kit and should be stored at 4°C in the sealed foil pouch with a desiccant pack.
8. **Rat Microalbumin Calibrator**
   The **lyophilized** Rat Microalbumin Calibrator should be stored at 4°C or frozen until reconstituted. The **reconstituted** calibrator should be aliquoted and stored frozen. Avoid multiple freeze/thaw cycles. **The working calibrator solutions should be prepared immediately prior to use.**
9. **Positive Control**
   Storage for less than 7 days can be at 4°C. For storage longer than 7 days keep frozen until the expiration date. Avoid multiple freeze/thaw cycles. It is not recommended to store this product in a frost-free freezer.

SPECIMEN COLLECTION AND HANDLING

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing.

If blood samples are clotted, grossly hemolyzed, lipemic, or the integrity of the sample is of concern, make a note and interpret results with caution.

The sample collection and storage condition listed below are intended as general guidelines. Sample stability has not been evaluated.

- **Serum samples** - Blood should be collected by venipuncture. The serum should be separated from the cells after clot formation by centrifugation. Remove serum and assay immediately or aliquot and store samples at -80˚C (preferably) or -20˚C. Avoid multiple freeze-thaw cycles
- **Plasma samples** - Blood should be collected into a container with an anticoagulant and then centrifuged. Assay immediately or aliquot and store samples at -80°C (preferably) or -20˚C. Avoid multiple freeze-thaw cycles.

- **Urine samples** - Collect mid-stream using a sterile or clean urine collector. Centrifuge to remove cell debris. Assay immediately or aliquot and store samples at -80°C (preferably) or -20˚C. Avoid multiple freeze-thaw cycles.
- **Known interfering substances** - Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.

REAGENT PREPARATION

1. **Bring all reagents to room temperature (RT) before use.**
2. **Diluent Concentrate**
   The Diluent solution supplied is a 5X concentrate and must be diluted 1:5 with distilled or de-ionized water. (1 part buffer concentrate, 4 parts dH_2O)
3. **Wash Solution Concentrate**
   The Wash Solution supplied is a 20X concentrate and must be diluted 1:20 with distilled or deionized water (1 part buffer concentrate, 19 parts dH_2O). Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.
4. **Enzyme-Antibody Conjugate Concentrate**
   Calculate the required amount of working conjugate solution for each microtiter plate test strip by adding 10 µL Enzyme-Antibody Conjugate to 990 µL of 1X Diluent for each test strip to be used for testing. Dilute immediately before use and protect from light. Mix uniformly, but gently to avoid forming foam.
5. **TMB Substrate Solution**
   Ready to use as supplied.
6. **Stop Solution**
   Ready to use as supplied.
7. **Microtiter Plate**
   Ready to use as supplied. Unseal the microtiter pouch and remove the plate from the pouch. Remove all of the strips and wells that **will not** be used in the assay and place back in pouch and re-seal along with desiccant.
8. **Rat Microalbumin Calibrator**
   Add **1.0 mL** of distilled or deionized water to the **lyophilized Rat Microalbumin Calibrator** and mix gently until dissolved. The calibrator is now at a concentration of **240.0 µg/mL** (*the reconstituted calibrator should be aliquoted and frozen if future use is intended*). Prepare the Rat Microalbumin Calibrators immediately prior to use according to the table below. Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent →	Volume of 1X Diluent
A	2,400	5 µL Rat Microalbumin Calibrator	495 µL
7	400	150 µL Calibrator A	750 µL
6	200	300 µL Calibrator 7	300 µL
5	100	300 µL Calibrator 6	300 µL
4	50	300 µL Calibrator 5	300 µL
3	25	300 µL Calibrator 4	300 µL
2	12.5	300 µL Calibrator 3	300 µL
1	6.25	300 µL Calibrator 2	300 µL
0	0		600 µL

9. **Positive Control**
   The concentration and recommended dilution are provided on the **Control Certificate**. Before use, briefly centrifuge the **Positive Control** to allow all of the liquid to collect in the bottom of the vial.

ASSAY PROTOCOL

Dilution of Samples

The assay for quantification of Microalbumin in samples requires that each test sample be diluted before use. All samples should be assayed in **duplicate** each time the assay is performed. The recommended dilutions are only suggestions. Dilutions should be based on the expected concentration of the unknown samples such that the diluted sample falls within the dynamic range of the calibration curve. For absolute quantification of samples that yield results outside the range of the calibration curve, a lesser or greater dilution might be required. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended**.

- **Serum and Plasma samples** - Recommended starting dilution is **1:1,000,000**. To prepare a 1:1,000,000 dilution of a sample, transfer 2 µL of sample to 1,998 µL of 1X diluent. This gives you a 1:1,000 dilution. Next dilute the 1:1,000 dilution by transferring 2 µL into 1998 µL of 1X diluent. This gives you a 1:1,000,000 dilution. Thoroughly mix at each stage.

- **Urine samples**- Recommended starting dilution is **1:500**. To prepare a 1:500 dilution of a sample, transfer 2 µL of sample to 998 µL of 1X diluent. This gives you a 1:500 dilution. Thoroughly mix at each stage.

Procedure

1. **All samples and Calibrators should be assayed in <u>duplicate</u>.**
2. The Calibrators and the test sample(s) should be loaded into the ELISA wells as quickly as possible to avoid a shift in optical density (OD) readings. Using a multichannel pipette would reduce this occurrence.

   Pipette 100 µL of

Calibrator 0	(0.0 ng/mL) in duplicate
Calibrator 1	(6.25 ng/mL) in duplicate
Calibrator 2	(12.5 ng/mL) in duplicate
Calibrator 3	(25 ng/mL) in duplicate
Calibrator 4	(50 ng/mL) in duplicate
Calibrator 5	(100 ng/mL) in duplicate
Calibrator 6	(200 ng/mL) in duplicate
Calibrator 7	(400 ng/mL) in duplicate

3. Pipette 100 µL of **diluted Positive Control** (in duplicate) into pre-designated wells.
4. Incubate the **Microtiter Plate** at RT for **thirty** (30 ± 2) minutes. Keep the plate covered and level during incubation.
5. Following incubation, aspirate the contents of the wells.
6. Completely fill each well with appropriately diluted **Wash Solution** and aspirate. Repeat three times, for a total of four washes. If washing manually; completely fill wells with diluted **Wash Solution**, invert the plate and pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual **Wash Solution**. Repeat 3 times for a total of 4 washes.
7. Pipette 100 µL of appropriately diluted **Enzyme-Antibody Conjugate** to each well. Incubate at RT for **thirty** (30 ± 2) minutes. Keep the plate covered in the dark and level during incubation.
8. Wash and blot the wells as described in Steps 5 and 6.

9. Pipette 100 µL of **TMB Substrate** Solution into each well.
10. Incubate in the dark at **RT** for precisely **ten** (10) minutes.
11. After **ten** (10) minutes, add 100 µL of **Stop Solution** to each well.
12. Determine the absorbance (at 450 nm) of the contents of each well within **thirty** (30) minutes. Calibrate the plate reader to the manufacturer's specifications.

RESULTS

1. Subtract the average background value (average absorbance reading of Calibrator 0) from the test values for each sample.

2. Average the duplicate readings for each calibrator and use the results to construct a Calibration Curve. Construct the curve by reducing the data using computer software capable of generating a four-parameter logistic curve fit. A second order polynomial (quadratic) or other curve fits may also be used; however, they will be a less precise fit of the data.

3. Interpolate test sample values from calibration curve. Correct for sample dilution factor to arrive at the Beta 2-Microglobulin concentration in original sample.

FOR RESEARCH USE ONLY

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