For Research Use Only

December 10, 2012 Ver. 6

[Mouse Albumin ELISA Kit]

(Code No.:AKRAL-121)

Please, read this instruction carefully before use.

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to Key points for ELISA by movie on our website:
http://www.shibayagi.co.jp/index-E.htm

1. Intended use

Mouse Albumin ELISA Kit is a sandwich ELISA system for quantitative measurement of mouse albumin. This is intended for research use only.

2. Storage and expiration

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Opened reagents should be used as soon as possible to avoid loss in optimal assay performance caused by storage environment.

3. Introduction

Albumin is mostly a simple hydrophilic protein present in cells and body fluids. Albumin is synthesized in the liver, and serum albumin (69 kDa, pI 4.9) occupies 56-60% of total serum proteins. Because of its large population, albumin is very important in maintaining plasma osmotic pressure. Albumin can bind hydrophobic physiological substances e.g. fatty acids, bilirubin, and thyroxine and contributes the transfer of these substances. Concentration of albumin in serum is lowered in liver cirrhosis, malnutrition, and pyrexial diseases due to decreased biosynthesis or increased consumption of albumin in, and also by secretion into urine in renal damage. In normal human subjects, excretion of albumin into urine is very little, about 30mg/day, but increased in glomerulonephritis, nephritic syndrome, and diabetic nephropathy. Urinary albumin sometimes increases in pyrexia, hypertension, congestive heart failure (CHF), and urinary tract infection (UTI). Even in healthy human, a transient upraise of urinary albumin is observed after hard exercise, muscle work, bathing in high temperature water, mental excitement, stress, intake of much protein, and before menstruation. These are called physiological or functional or sports proteinuria (albuminuria). Orthostatic proteinuria (albuminuria) is observed in teenagers. Rare hereditary analbuminemia in human is really albumin deficiency.. A model analbuminemia rat has been established by Dr. Sumi Nagase which has been derived from Sprague-Dawley strain, and is called Nagase analbuminemia rat (NAR). Routine measurement of serum albumin is conveniently made with Shibayagi's TIA assay kits for automatic analyzers. Shibayagi's Albumin ELISA Kits can measure urinary albumin with high sensitivity, and are also applied to *in vitro* albumin biosynthesis system, checking of contamination with albumin in biological active substance preparations obtained by culture system, and liver transplantation experiments using NAR.

4. Assay principle

In Shibayagi's Mouse Albumin ELISA Kit, standards or diluted samples are incubated in monoclonal anti-albumin antibody-coated wells to capture albumin. After 1 hour incubation and washing, HRP (horse radish peroxidase)-conjugated anti-albumin antibody is added, and incubated for 1 hour. After washing, HRP-complex remaining in wells is reacted with a chromogenic substrate (TMB) for 20 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is nearly proportional to albumin concentration. The standard curve is prepared by plotting absorbance against standard albumin concentrations. Albumin concentrations in unknown samples are determined using this standard curve.

5. Precautions

- For professional use only. Beginners are advised to use this kit under the guidance of experienced person.
- Do not drink, eat or smoke in the areas where assays are carried out.
- This kit contains components of animal origin. These materials should be handled as potentially infectious.
- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1 M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate reagent containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Unused samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate seal supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. ① (including wind from air conditioner), and humidity less than 30%. ①For airstream, refer to [Assay circumstance] on our web site.

6. Reagents supplied

Components	State	Amount
(A) Anti-albumin-coated plate	Ready for use.	96 wells/1 plate
(B) Standard mouse albumin (10 μg/ml) (derived from mouse)	Concentrated. Use after dilution.	150 μl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) HRP-conjugated anti-albumin antibody	Concentrated. Use after dilution.	100 μl/1 vial
(F) Chromogenic substrate reagent (TMB)	Ready for use.	12 ml/1 bottle
(H) Reaction stopper (1M H_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
(I) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal	—	3 sheets
Instruction Manual	—	1 copy

7. Equipments or supplies required but not supplied ☐Use as a check box

☐Purified water (distilled water)
☐Test tubes for preparation of standard solution series.
☐Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
☐Pipettes (disposable tip type). One should be able to deliver 5 μl precisely, and another for 50-500 μl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 μl.
☐Paper towel to remove washing buffer remaining in wells.
☐A vortex-type mixer.
☐A shaker for 96 well-plate (600-1200rpm)
☐An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle. (refer to our web movie [Washing of microplate]).

☐A 96 well-plate reader (450nm ±10nm, 620nm: 600-650nm)
☐Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

8. Preparation of reagents

◆Bring all reagents of the kit to room temperature (20-25 °C) before use.
◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(B) Standard mouse albumin (10 μg/ml)]

Below is an example of preparing each standard solution.

Volume of standard solution	Buffer solution	Concentration(ng/ml)
Original solution 50 μl	450 μl	1000
1000 ng/ml solution 400 μl	100 μl	800
800 ng/ml solution 300 μl	100 μl	600
600 ng/ml solution 200 μl	100 μl	400
400 ng/ml solution 100 μl	100 μl	200
200 ng/ml solution 100 μl	100 μl	100
100 ng/ml solution 100 μl	100 μl	50
Blank	100 μl	0

[(D) HRP-conjugated anti-albumin antibody]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

[(I) Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of dionized water.

【Storage and stability】

[(A) Anti-albumin-coated plate]

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[(B) Standard mouse albumin (10 μg/ml)]

Standard solutions prepared above should be used as soon as possible, and should not be stored. Dispose remaining prepared solution.

[(C) Buffer solution] and [(F) Chromogenic substrate reagent]

Use only volume you need for your assay. Remaining reagents should be stored at 2-8 °C fastening the cap tightly. It maintains stability until expiration date. Once opened, we recommend using as soon as possible to avoid influence by environmental condition.

[(D) HRP-conjugated anti-albumin antibody]

Unused working solution (already diluted) should be disposed.
The rest of the undiluted solution: if stored tightly closed at 2-8 °C, it is stable until expiration date.

[(H) Reaction stopper (1 M H_2SO_4)]

Close the stopper tightly and store at 2-8 ° C. It maintains stability until expiration date.

[(I) Concentrated washing buffer (10x)]

The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date. Dispose any unused diluted buffer.

9. Technical tips

- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if

the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.

- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution. Otherwise, store them in a dark place at 2-8 ºC.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic sucstrate reagent (TMB) should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the antibody-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

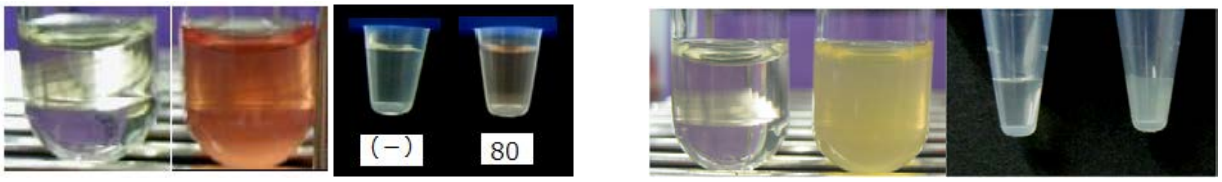
10. Preparation of samples

This kit is intended to measure albumin in mouse serum, plasma (heparin is recommended) or urine. The necessary sample volume for the standard procedure is 5 µl. Dilute your samples with the buffer so as to be within the assay range, 50-1,000 ng/ml. **The recommended dilution is 10,000-50,000x for serum or plasma, and 100x for urinary samples.**

Samples should be immediately assayed or stored below –35 ºC until assay. Before starting assay, shake thawed samples sufficiently. Do not repeat freeze-and-thaw cycles.

Hemolytic and hyperlipemic serum samples are not suitable.

* To avoid influence of blood (high lipid or hemolysis, etc.), if your original samples have heavy chyle or hemolysis as the pictures below, do not use them for assay. Abnormal value might be obtained with hemolysis above 80mg/dL with this kit.



Normal | Hemolysis 80mg/dL | normal | Hemolysis 80mg/dL | Normal | Chyle Highly lipid sample | Normal | Chyle Highly lipid sample

If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Dilution of a sample should be made in a test tube using buffer solution prior to adding them to wells.

Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

●Testing for compatibility of your samples with Shibayagi's kit using a positive sample.

Due to various factors of your sampling conditions (anesthesia, preservatives, anticoagulants, raised sample pH caused by loss of CO_2 during standing and storage, preservative used, evaporation and condensation during storage in a freezer, etc), sometimes the kit does not work well with your samples. If the standard curve is in a good shape, while your samples give low absorbance, please check the compatibility of your samples (serum or plasma) by a simple recovery test as follows.

Place 90 µl of your already diluted sample (e.g. a sample from control group in your experiment) in a small test tube, then add 10 µl of the highest standard solution. Assay this mixture together with the original sample, and compare the assay values. The assay value of the mixture will be

around [0.9 x original sample + 0.1 x highest standard concentration]. If the assay value is increased as expected, the assay system is working well with your sample.

Especially when you use Shibayagi's kit for the first time, we recommend you to run this simple recovery test.

●Quality control samples

We recommend preparing quality control samples of your own laboratory by storing many aliquots of serum, plasma or culture medium with known amount of the analyte to be measured after initial testing. Keep them in small and tightly capped sample tubes below −35 °C. If the sample tube is too big, water will be lost during storage. If possible, prepare high and low controls.

Measure these control samples along with your samples in every run to confirm the reproducibility and successful performance of the assay system.

11. Assay procedure

Remove the cover sheet of the antibody-coated plate after bringing up to room temperature.

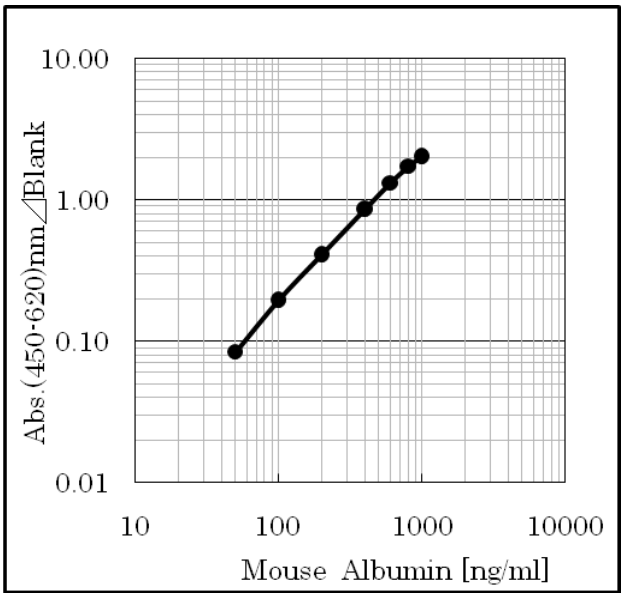
(1) Wash the anti-albumin-coated plate (A) by filling the wells with washing buffer and discard 3 times(*②), then strike the plate upside-down onto folded several sheets of paper towel to remove residual buffer in the wells.
(2) Pipette 50 µl of buffer to each well and shake the plate gently on a plate shaker(*③).
(3) Pipette 5 µl of standard solution to the wells designated for standards, and 5 µl of properly diluted samples to the designated sample wells. .
(4) Shake the plate gently on a plate shaker(*③).
(5) Stick a plate seal (*④) on the plate and incubate for 1 hour at 20-25°C.
(6) Discard the reaction mixture and rinse wells as step (1).
(7) Pipette 50µl of HRP-conjugated anti-albumin antibody to all wells, and shake as step (4).
(8) Stick a plate seal (*④) on the plate and incubate the plate for 1 hour 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 50µl of Chromogenic substrate reagent to wells, and shake as step (4).
(11) Stick a plate seal (*④) on the plate and incubate the plate for 20 minutes at 20-25°C.
(12) Add 50 µl of the reaction stopper to all wells to stop the coloration.
(13) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

*Refer to the page 7-8 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve by plotting standard concentration on X-log and absorbance on Y-axis.
(Refer to our web site for more detailed explanation about standard curve. Shibayagi is offering a convenient Excel template. http://www.shibayagi.co.jp/en/tech_003.html)

(2) Using the standard curve, read the albumin concentration of a sample at its absorbance*, and multiply the assay value by dilution factor if the sample has been diluted. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution.



Mouse Albumin assay standard curve (an example)
Absorbance may change due to assay environment.

* We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.
Physiological or pathological situation of mouse should be judged comprehensively taking other examination results into consideration.

13. Performance characteristics

- Assay range

The assay range of the kit is 50 ~ 1000 ng/ml.

- Specificity

The antibodies used in this kit are specific to albumin.

Sample	Cross reaction
Rat albumin	Less than 5%
Human albumin	N.D.
Porcine albumin	N.D.
Bovine albumin	N.D.
10% FCS	N.D.

*Cross reaction at 10,000 ng/ml (Except FCS)

- Precision of assay

Within assay variation (3 samples, 5 replicates assay), Mean CV is less than 5%

- Reproducibility

Between assay variation (3 samples, 3 days, 3 replicates assay), Mean CV is less than 5%

- Recovery test

Standard albumin was added in 3 concentrations to serum sample and assayed.
The recoveries were 94.3 ~104%

- Dilution test

Serum sample was serially diluted by 3 steps.
The dilution curves showed linearity with $R^2 = 0.999$.

14. Trouble shooting

- Low absorbance in all wells

Possible explanations:

1) The standard or samples might not be added.
2) Reagents necessary for coloration such as HRP-conjugated anti-albumin antibody or Chromogenic substrate reagent might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of HRP-conjugated anti-albumin antibody.
4) Contamination of enzyme inhibitor(s).
5) Influence of the temperature under which the kits had been stored.
6) Excessive hard washing of the well plate.
7) Addition of chromogenic substrate reagent soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

- Blank OD was higher than that of the lowest standard concentration (50 ng/ml).

Possible explanations:

Improper or inadequate washing. (Change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP-conjugated anti-albumin antibody.)

- High coefficient of variation (CV)

Possible explanation:

1) Improper or inadequate washing.
2) Improper mixing of standard or samples.
3) Pipetting at irregular intervals.

- Q-1: Can I divide the plate to use it for the other testing?

A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon

- Q-2: I found there contains liquid in 96 well-plate when I opened the box. What is it?

A-2: When we manufacture 96 well-plate, we insert preservation stabilizer in wells.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits"** on our website (http://www.shibayagi.co.jp/en/tech_004.html).

15. References

Please, refer to [User's Publication] on our website.

Summary of assay procedure ☐ : Use as a check box

*First, read this instruction manual carefully and start your assay after confirmation of details.

For more details, watch our web movie [ELISA by MOVIE] on our website.

☐Bring the well-plate and all reagents back to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified water that returned to 20-25°C.

☐Standard solution dilution example:

Concentration (ng/ml)	1000	800	600	400	200	100	50	0
Standard solution (µl) orig.sol.	50	400*	300*	200*	100*	100*	100*	0
Buffer solution (µl)	450	100	100	100	100	100	100	100

*One rank higher standard.

☐Preparation of the positive sample and samples.

			Precautions & related info
☐	Anti-albumin-coated plate		
☐	↓ Washing 3 times(*②)		*⑥
☐	Buffer	50 µl	
☐	↓ Shaking*(*③)		
☐	Diluted samples, or Standards	5 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute HRP-conjugated anti-albumin antibody (D) to 100x with buffer (C) returned to 20-25°C.		
☐	↓ Washing 3 times(*②)		*⑥
☐	HRP-conjugated anti-albumin antibody	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓ Washing 3 times(*②)		*⑥
☐	Chromogenic substrate reagent (TMB)	50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 20 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	50 µl	After dispense, the color turns to yellow depending on the concentration.
☐	↓ Shaking(*③)		Immediately shake.
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤))		Ref. wave cancels the dirt in the back of plate.

*②After dispensing wash buffer to wells, lightly shake the plate on your palm for 10 sec and remove the buffer. Guideline of washing volume: 300µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP conjugated streptavidin.
Standard of plate-washing pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.

*④Seal the plate during the reaction after shaking. Peel off the protective paper from the seal

and stick the seal on the plate. Do not reuse the plate seal used once.
*⑤600-650 nm can be used as reference wavelength.
*⑥After removal of wash buffer, immediately dispense the next reagent.
*⑦Refer to our web movie [Handling of pipetting].
*⑧Refer to our web movie [Assay circumstance].

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	**1000 ng/ml**	Pos.Control.	Sample 8	Sample 16	Sample 24	Sample 32
B	**800 ng/ml**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	**600 ng/ml**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	**400 ng/ml**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	**200 ng/ml**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	**100 ng/ml**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	**50 ng/ml**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	**0**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

Assay worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

[Storage condition] Store the kit at 2-8°C (Do not freeze).
[Term of validity] 6 months from production (Expiration date is indicated on the container.)

Shibayagi