For Research Use Only December 3, 2012 Ver. 5

[Mouse/Rat HMW Adiponectin ELISA Kit]

(Code No.:AKMAN-011)

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/Rat HMW Adiponectin ELISA Kit is a sandwich ELISA system for quantitative measurement of mouse or rat High Molecular Weight Adiponectin. This is 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. Reagent, once opened, should be used as soon as possible to avoid losing its optimal assay performance caused by storage environment.

3. Introduction

Adiponectin is one of cytokines secreted from adipocyte (adipocytokines, adipokines), and controls lipid metabolism and insulin sensitivity. Adiponectin is an important substance showing anti-diabetic, anti-atherosclerotic, and anti-inflammatory actions. In blood, adiponectin monomer molecules are associated to form trimer, hexamer or 12-18mers. The monomer form of adiponectin is found only in adipocytes, and homomultimer, i.e. trimer, hexamer and 12-18-mers formed from adiponectin monomer are found in plasma.

Trimer (low molecular weight complexes/LMW) is formed by interaction of non-covalent bonds of triple helix area and by hydrophobic interaction between globular Cq1 domain, and hexamer (middle molecular weight complexes/MMW) or larger complexes (higher molecular weight/HMW) are formed through disulfide bonds between cysteines at 39 of LMW-complexes.

Adiponectin is believed to influence on cell growth, angiogenesis, and tissue remodeling by isolating various growth factors through binding with distinct affinity. The affinity is dependent upon the type of complexes, LMW, MMW and HMW.

The blood levels of HMW were reported to reflect BMI, sex, effect of body weight decrease, glucose tolerance, liver insulin sensitivity, metabolic syndrome or DM2 more than total adiponectin levels. This means that assay for HMW is expected to be more useful for analysis of metabolic syndrome and DM2. Shibayagi's Mouse/Rat HMW Adiponectin ELISA KIT is specific to HMW.

4. Assay principle

In Shibayagi's Mouse/Rat HMW adiponectin ELISA Kit, standards or diluted samples are incubated in monoclonal anti-adiponectin antibody-coated wells to capture HMW adiponectin. After 2 hours incubation and washing, HRP (horse radish peroxidase)-conjugated anti-adiponectin antibody is added, and incubated for 90 minutes. After washing, HRP-complex remaining in wells is reacted with a chromogenic substrate (TMB) for 30 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 HMW adiponectin concentration. The standard curve is prepared by plotting absorbance against standard HMW adiponectin concentrations. HMW adiponectin 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.
- Wear gloves and laboratory coats when handling assay materials.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- 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 solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Residual 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) Antibody-coated 96 well-plate	Use after washing	96 wells/1 plate
(B) Standard Adiponectin (2000 ng/ml) (derived from mouse)	Concentrated. Use after dilution	200 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) HRP-conjugated anti-adiponectin antibody	Concentrated. Use after dilution.	100 µl/1 vial
(F) Chromogenic substrate (TMB) solution	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 5-10 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 (600-1,200rpm)
☐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 Adiponectin (2000 ng/ml)]

Make a serial dilution of original standard solution to prepare each standard solution. An example is shown below.

Volume of standard solution	Buffer solution	Concentration(ng/ml)
Original solution : 50 µl	450 µl	200
200 ng/ml solution : 200 µl	200 µl	100
100 ng/ml solution : 200 µl	200 µl	50
50 ng/ml solution : 200 µl	200 µl	25
25 ng/ml solution : 200 µl	200 µl	12.5
12.5 ng/ml solution : 200 µl	200 µl	6.25
6.25 ng/ml solution : 200 µl	200 µl	3.13
0 (Blank)	200 µl	0

[(D) HRP-conjugated anti-adiponectin 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 deionized water.

【Storage and stability】

[(A) Antibody-coated well-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 Adiponectin (2000 ng/ml)]

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

[(C) Buffer solution] & [(F) Chromogenic substrate solution]

If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using them as soon as possible to avoid influence by environmental condition.

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

Unused working solution (already diluted) should be disposed.

[(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 °C.
- 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 substrate (TMB) solution 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].

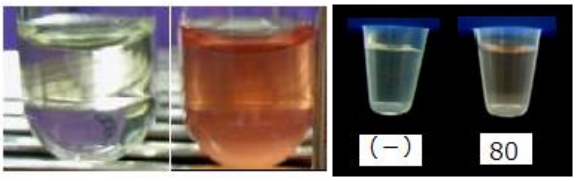
10. Preparation of samples

This kit is intended to measure HMW Adiponectin in mouse or rat serum, plasma or culture medium. The necessary sample volume for the standard procedure is 10 µl.

Samples should be immediately assayed or stored below –35 °C for several days. Defrosted samples should be mixed thoroughly for best results.

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. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter. Sample dilution should be carried out with the buffer solution (C) using small test tube such as PP, PE or glass, before assay. You can choose dilution rate 25-50x if necessary.

Example of dilution:	Rate	50x	(25x)
	Sample (µl)	10	(10)
	Buffer (µl)	490	(240)

Storage and stability

Adiponectin in samples will be inactivated if stored at 2-8 °C. If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Avoid repeated freezing and thawing.

11. Assay procedure

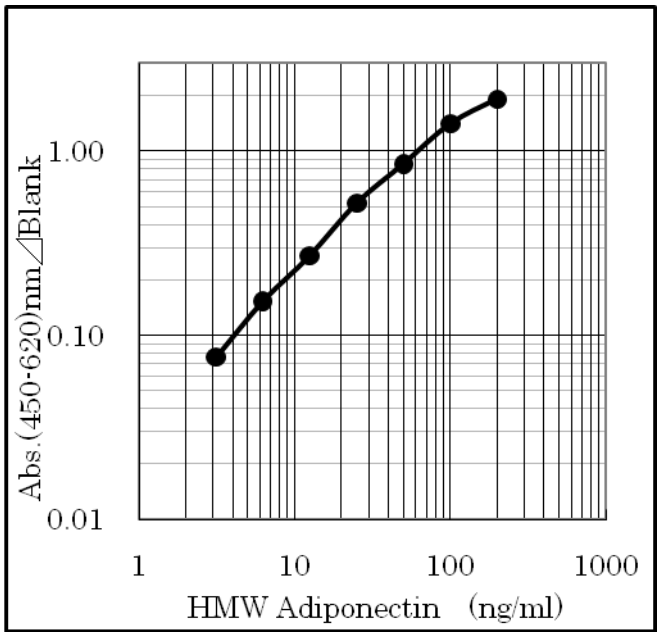
Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

(1) Wash the anti-adiponectin coated plate (A) by filling the well with washing buffer and discard 3 times(*②), then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
(2) Pipette 50 µl of diluted samples to the designated sample wells.
(3) Pipette 50 µl of standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker (*③)
(5) Stick a plate seal (*④) on the plate and incubate for 2 hours at 20-25°C.
(6) Discard the reaction mixture and rinse wells as step (1).
(7) Pipette 50 µl of HRP-conjugated anti-adiponectin antibody solution to all wells, and shake as step (4).
(8) Stick a plate seal (*④) on the plate and incubate the plate for 90 minutes at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 50 µl of chromogenic substrate solution to wells, and shake as step (4).
(11) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(12) Add 50 µl of the reaction stopper to all wells and shake as step (4).
(16) 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 using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against HMW adiponectin concentration (ng/ml) on X-axis. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.
(2) Using the standard curve, read the HMW adiponectin 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. * We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.



HMW adiponectin assay standard curve (an example)
Absorbance may change due to assay environment.

(3) **Assay results from this product and results from other commercially available mouse/rat (total) adiponectin ELISA kits cannot be compared because of the difference of the assay systems.**

13. Performance characteristics

● Assay range

The assay range of the kit is 3.13 ~ 200 ng/ml.

● Specificity

The kit uses two monoclonal antibodies specific to mouse/rat HMW adiponectin.

Sample	Cross reaction	Sample	Cross reaction
Mouse adiponectin (HMW)	100%	Rat adiponectin (HMW)	100%
Mouse adiponectin (Hexamer)	<5%	Tested at 1000 ng/ml.	

No crossreaction at 1000 ng/ml: Mouse adiponectin (Trimer), Mouse adiponectin (Monomer), Mouse MCH, Mouse TNF-α, Mouse INF-γ, Mouse Insulin, Mouse Leptin, Rat adiponectin (Monomer), Rat TNF-α, Rat INF-γ, Rat Insulin, Rat Leptin

● Precision of assay

Within assay variation (2 samples, 5 replicates assay,) Mean CV was less than 5 %.

● Reproducibility

Between assay variation (3 samples, 4 days, duplicate assay) Mean CV was less than 5 %

● Recovery test

HMW Adiponectin was added in 3 concentrations to 2 serum samples and was assayed.
The recoveries were 94.4 ~ 105%

● Dilution test

2 serum samples were serially diluted by 3 steps.
The dilution curves showed excellent linearity. (R^2= 0.999)

14. Reference assay data

Strain	Week	Gender	numbers	Results (ng/ml)		Remarks
				Mean	SD	
BALB/c	6w	♂	10	2,369	743	Serum, ad libitum feeding
ICR	6w	♂	10	2,119	802	Serum, ad libitum feeding
CD	8w	♂	9	3,220	670	Serum, ad libitum feeding

These data should be considered as guidance only. Each laboratory should establish its own normal and pathological reference ranges for HMW Adiponectin levels independently.

15. 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-body, or TMB might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-labeled antibody or HRP-conjugated 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 TMB solution soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

● The OD of blank is higher than that of the lowest standard concentration (3.13 ng/ml)

Possible explanations:

Improper or inadequate washing. (Change washing repetition from 3 times to 4-6 times after the reaction with HRP-labeled 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 put protective solution 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).

16. 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 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 adiponectin solution dilution example:

Concentration (ng/ml)	200	100	50	25	12.5	6.25	3.13	0
Standard solution (µl) Orig.sol.	50	200*	200*	200*	200*	200*	200*	0
Buffer solution (µl)	450	200	200	200	200	200	200	200

*One rank higher standard.

☐Make the positive control.

			Precautions & related info
☐	Antibody-coated 96 well-plate		
☐	↓ Washing 3 times(*②)		*⑥
☐	Diluted Samples / Standards	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	HRP-conjugated anti-adiponectin antibody (D) Dilute to 100 times by using buffer solution(C) and use.		Dilute reagents during the first reaction.
☐	↓ Washing 3 times(*②)		*⑥
☐	HRP-conjugated anti-adiponectin antibody	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 90 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓ Washing 3 times(*②)		*⑥
☐	Chromogenic substrate (TMB)	50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1 M 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	200 ng/ml	Pos. Control	Sample 8	Sample 16	Sample 24	Sample 32
B	100 ng/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	50 ng/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	25 ng/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	12.5 ng/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	6.25 ng/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	3.13 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.)

This kit is manufactured by **Shibayagi Co., Ltd.**
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