For Research Use Only

October 31, 2014 Ver. 5

[Mouse Leptin ELISA Kit]

(Code No.: AKRLP-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 Leptin ELISA Kit is a sandwich ELISA system for quantitative measurement of mouse leptin. This is intended for research use only.

2. Storage and expiration

When the intact kit is stored at 2-8°C (Do not freeze), the kit is stable until the expiration date shown on the label on the container (6 months from production). Opened reagents should be used as soon as possible to avoid losing its optimal assay performance caused by storage environment.

3. Introduction

Leptin is a 16kDa protein mostly produced in white fat tissue, and also expressed in brown fat tissue, placenta, ovary, skeletal muscle, fundus of the stomach, epithelial cells of mammary gland, bone marrow, pituitary gland, and liver. Leptin was first found in mutant obese mouse and proved to be an important hormone relating to energy balance through controlling apetite and metabolism. In amino acid sequence of leptin, 97% homology is observed between mouse and rat. Blood leptin level is decreased in fasting or by low calorie meal. Postprandial increase of blood leptin level is not evident, however, it rises in obesity or proliferation of adipose tissue reflecting the volume of body fat. Leptin enters the brain and suppresses NPY/AgRP-expressing neuron and activates α-MSH-expressing neuron, causing loss of appetite and food intake. However, highly obese patients, though their blood levels of leptin are very high, show leptin-resistance and the anorexic effect of leptin does not work. Lowered production and deficiency of leptin cause insulin resistance, diabetes mellitus, fatty liver, and hypertriglyceridemia. In addition, leptin is considered to be involved in immune reaction to atherosclerosis, sexual cycle-related energy balance, and surfactant production in alveoli of the lung.

4. Assay principle

In Shibayagi's Mouse Leptin ELISA Kit, biotin-conjugated anti-leptin, and standard or sample are incubated in monoclonal anti-leptin-coated wells to capture leptin bound with biotin-conjugated anti-leptin. After 2 hours' incubation and washing, Peroxidase-conjugated streptavidin is added, and incubated for 30 minutes. After washing, Peroxidase-conjugated streptavidin remaining in wells are reacted with a substrate chromogen reagent (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 proportional to leptin concentration. The standard curve is prepared by plotting absorbance against the standard concentrations. Leptin 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. In manual operation, proficiency in pipetting technique is recommended.
- Use clean laboratory glassware.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- 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 1M 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.
- 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.
●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 egional/national regulatory requirements.
●The materials must not be pipetted by mouth.
●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-leptin-coated plate (Dried-plate)	Use after washing	96 wells/1 plate
(B) Standard mouse leptin solution (5,000 pg/ml) (derived from mouse)	Concentrated. Use after dilution	500 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-conjugated anti-leptin	Concentrated. Use after dilution.	200 µl/1 vial
(E) Peroxidase-conjugated streptavidin	Concentrated. Use after dilution.	200 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 10-50 µl precisely, and another for 50-400 µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 µl and 100 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 mouse leptin solution (5,000 pg/ml)]

Make a serial dilution of master standard (5,000 pg/ml) solution to prepare each standard solution. Example is shown below.

Volume of standard solution	Buffer solution	Concentration (pg/ml)
Original solution	-	5,000
Original solution: 100 µl	200 µl	1,667
1,667 pg/ml solution: 100 µl	200 µl	556
556 pg/ml solution: 100 µl	200 µl	185
185 pg/ml solution: 100 µl	200 µl	61.7
61.7 pg/ml solution: 100µl	200 µl	20.6
0 (Blank)	200 µl	0

[(D) Biotin-conjugated anti-leptin]

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

[(E) Peroxidase-conjugated streptavidin]

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

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

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

【Storage and stability】

[(A)Anti-leptin-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 leptin solution (5,000 pg/ml)]

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

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

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

[(D) Biotin-conjugated anti-leptin] & [(E) Peroxidase-conjugated streptavidin]

Unused working solution (already diluted) should be disposed.

[(H) Reaction stopper (1M 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

- 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.
- 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.
- 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.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic substrate reagent (TMB) should be almost colorless or clear pale yellow before

use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.

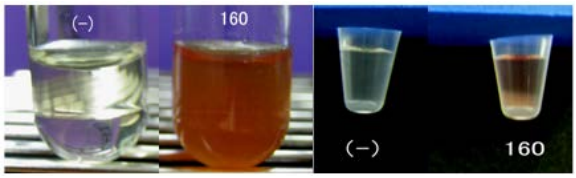
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- When ELISA has to be done under the airstream velocity over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

10. Preparation of samples

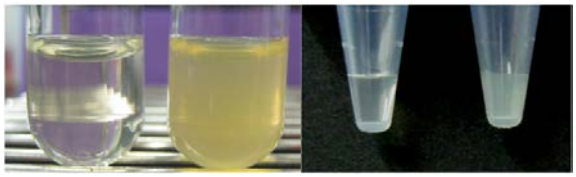
This kit is intended to measure leptin in mouse serum and plasma. The necessary sample volume for the standard procedure is 10 µl. Samples should be immediately assayed or stored below –35 °C for several days. Defrosted samples should be mixed thoroughly for best results. Avoid repeated freeze-thaw cycles.

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 160mg/dL with this kit.



Normal | Hemolysis 160mg/dL | normal | Hemolysis 160mg/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.

Storage and stability

Leptin in samples will be inactivated if stored at 2-8ºC. If you have to store samples for a longer period, snap-freeze samples and keep them below –35ºC. Avoid repeated freeze-thaw cycles. Dilute samples just before starting assay.

11. Assay procedure

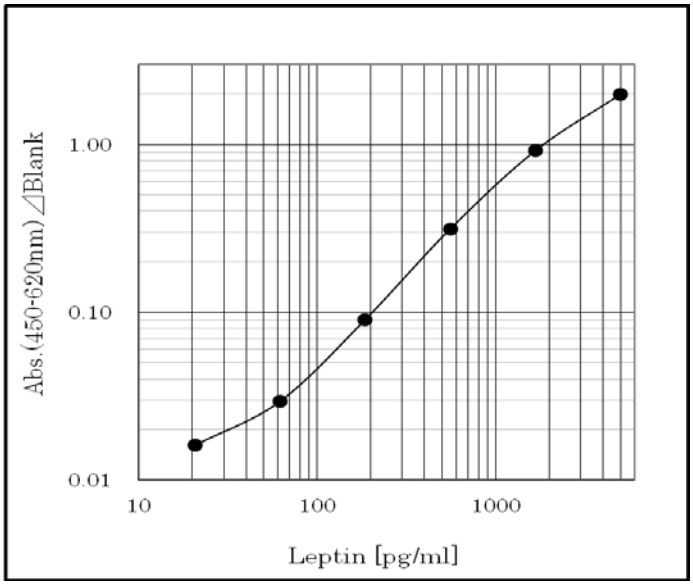
Remove the cover sheet of the anti-leptin-coated plate after bringing up to 20-25ºC.

(1) Wash the anti-leptin-coated plate (A) by filling the wells with 300 µl of washing buffer and discard 4 times(*②), then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(2) Pipette 40µl of buffer solution (C) into the wells for samples, and add 10µl of sample to each well. Sample volume can be adjusted within 10-50µl, but total volume to add to each well should be 50µl.
(3) Pipette 50µl of standard solution to the wells designated for standards..
(4) Shake the plate gently on a plate shaker(*③) .
(5) Pipette 50µl of biotin-conjugated anti-leptin to the wells. Shake the plate gently on a plate shaker(*③).
(6) Stick a plate seal (*④) on the plate and incubate for 2 hours at 20-25ºC.
(7) Discard the reaction mixture and rinse wells as step (1).
(8) Pipette 100µl of Peroxidase-conjugated streptavidin to all wells, and shake as step (5).
(9) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25ºC.
(10) Discard the reaction mixture, and then wash the plate as step (1).
(11) Pipette 100µl of Chromogenic substrate reagent to wells, and shake as step (5).
(12) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25ºC.

(13) Add 100 μl of the reaction stopper to all wells and shake as step (5).
(14) Measure the absorbance of each well at 450 nm (reference wavelength, 620nm) using a plate reader within 30 minutes. 600-650nm can be used as reference wavelength.
*Refer to the page 7-8 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve for each assay. Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against leptin concentration (pg/ml) on X-axis.
(2) Using the standard curve, read the leptin 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.
- ●Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.



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

13. Performance characteristics

- ●Assay range of the kit is 20.6-5,000 pg/ml (in case of 5x dilution, 103-25,000 pg/ml).
- ●Specificity: the antibodies used in this kit are specific to mouse leptin.

Cross-reactivity of the kit is shown below (concentration at 3,000 pg/ml).

Substances	Cross-reactivity	Substances	Cross-reactivity
Mouse Leptin	100%	Mouse TNF-α	-
Mouse α-MSH	-	Rat Leptin	31.5%
Mouse IFN-γ	-	Human Leptin	+
Mouse MCH	-	+:Cross reaction	-:No cross reaction

- ●Precision of assay

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

- ●Reproducibility

Between assay variation (2 samples, 4 days, duplicate assay); Mean CV is less than 5%

●Recovery test
Standard leptin was added in 3 concentrations to 2 serum samples and were assayed.
The recoveries were 95.0 ~105%
●Dilution test
2 serum samples were serially diluted by 4 steps.
The dilution curves showed linearity with $R^2 = 0.9992$~0.9997.

14. Reference assay data

Please contact to: syc-info@shibayagi.co.jp

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 biotin-conjugated anti-leptin, peroxidase-conjugated streptavidin, or chromogenic substrate reagent might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-conjugated anti-leptin or peroxidase-conjugated streptavidin.
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 (20.6 pg/ml).
Possible explanations: Improper or inadequate washing. (Change washing frequency from 4 times to 5-8 times at the constant stroke after the reaction with Peroxidase-conjugated streptavidin.)

●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 96 well-plate is empty when I opened the box.
A-2: As this kit is dried type, not preservation stabilizer is added.

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 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 mouse leptin dilution example:

Concentration (pg/ml)	5,000	1,667	556	185	61.7	20.6	0
Standar leptin (µl)	Orig. sol.	Orig.sol.100	100*	100*	100*	100*	0
Buffer solution (µl)		200	200	200	200	200	200

*One rank higher standard.

☐Biotin-conjugated anti-leptin (D) : Dilute to 100 times by using buffer solution(C).

			Precautions & related info
☐	Anti-leptin-coated plate (Dried-plate)		
☐	↓Washing 4 times(*②)		*⑥
☐	Diluted samples (e.g. buffer (C) 40µl + sample 10µl) or Standards	50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③)		
☐	Biotin-conjugated anti-leptin	50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Peroxidase-conjugated streptavidin (E) Dilute to 100 times by using buffer solution(C).		This should be prepared during incubation. Preparation volume should be twice of Biotin-conjugated anti-leptin.
☐	↓Washing 4 times(*②)		*⑥
☐	Peroxidase-conjugated streptavidin	100 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓Washing 4 times(*②)		*⑥
☐	Chromogenic substrate reagent (TMB)	100 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 (1M H_2SO_4)	100 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 4 times to 5-8 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	5,000 pg/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
B	1,667 pg/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
C	556 pg/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
D	185 pg/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
E	61.7 pg/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
F	20.6 pg/ml	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
G	0	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40
H	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41

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 2-8ºC (Do not freeze).
[Term of validity] 6 months from production (Expiration date is indicated on the container.)