YK050 **Rat Leptin ELISA**

I. Introduction

Leptin, which is a product of *ob* gene, is a protein consisting of 167 amino acids and it is secreted from white adipose tissue. It is known that leptin acts on hypothalamus to decrease food intake and to reduce body weight, body fat, blood sugar and blood insulin in a healthy and an *ob/ob* mouse. Further, gene expression of neuropeptide Y(NPY) is surpressed by leptin.

Recently, radioimmunoassay for leptin determination in human plasma has become available and leptin level in human patient group with obesity was found to increase in comparison with that of normal group.

The level well correlate with body fat and these observations show clearly that leptin concentration in human plasma reflects the tissue fat weight. The measurement of plasma leptin may be an excellent index of obesity.

Although rat leptin shows a high homology(96%) with mouse leptin, it is observed that substitution of several amino acid residues occurs at both end N- and C- terminal region between human and rat leptin. These findings have required urgently to develop highly sensitive immunoassay system specific to rat leptin.

Yanaihara Insutitute Inc. has developed the enzyme immunoassay (EIA) kit which is a stable and convenient assay system for rat leptin in its plasma, serum and culture supernatant.

II. Characteristics

This ELISA kit is used for quantitative determination of rat leptin in its plasma, serum & culture supernatant samples. It has a lot of advantage to perform the assay, such as good quantification, no influence with other body fluid factors or physiological active substances and needlessness of sample pretreatment.

< Specificity >

The EIA kit has high specificity to rat leptin and shows less than 0.02% – 0.04% cross reactivity to human Leptin. And it has no cross reactivity with rat IL-1α, IL-1β, rat TNF-α, human TNF-α and other cytokines.

< Test Principle >

This EIA kit for determination of rat leptin in plasma, serum and culture supernatant samples is based on a sandwich enzyme immunoassay. The 96 wells plate is coated with anti rat leptin monoclonal antibody. Rat leptin standard or samples and HRP-labeled anti rat leptin polyclonal antibody are added to the wells for one step sandwich immunoreaction. During this immunoreaction, monoclonal antibody – antigen –HRP labeled antibody complex are formed. After rinsing out excess HRP labeled antibody, HRP enzyme activity is determined and the concentration of rat leptin is calculated.

III. Composition

Component	Form	Quantity	Main Ingredient
1. Antibody coated plate	MTP*1	1 plate (96 wells)	Anti rat Leptin monoclonal antibody
2. Rat Leptin standard	lyophilized	1 vial	Rat Leptin (20ng)
3. HRP labeled antibody	liquid	1 bottle(6 mL)	HRP labeled antibody from rabbit
4. Substrate buffer	liquid	1 bottle (24 mL)	0.015% Hydrogen Peroxide
5. OPD tablet	tablet	2 tablets	o-Phenylenediamine hydrochloride
6. Stopping solution	liquid	1 bottle (12 mL)	2N-H_2SO_4
7. Buffer solution A	liquid	1 bottle (20 mL)	Phosphate buffer including animal serum
8. Buffer solution B	liquid	1 bottle (15 mL)	Phosphate buffer including surfactant
9. Washing solution (concentrated)	liquid	1 bottle (50 mL)	Concentrated saline
10. Adhesive foil		2 sheets	

MTP*1..... Microtiration plate

Ⅳ. Method

< Equipment required >

1) Photometer for microtitration plate(Plate reader),which can read extinction 2.5 at 490nm

2) Rotator for microtitration plate

3) Washing device for microtitration plate and dispenser for approximately 0.35 mL with aspiration system

4) Micropipettes, multi-channel pipettes for 8 wells or 12 wells and their tips

5) Test tubes for preparation of standard solution

6) Graduated cylinder(1,000 mL)

7) Distilled water or deionized water

< Preparatory work >

1) Preparation of standard solution:

(A)Sample volume 20μL(plasma and serum)

Reconstitute the Rat Leptin standard(lyophilized/20ng/vial) with 1mL of buffer solution A, which affords 20,000pg/mL standard solution. Then, 0.5ml of the standard solution is diluted with 0.5 mL of buffer solution A, that yields 10,000pg/mL standard solution. Repeat the dilution to make each standard of 5,000, 2,500, 1,250, 625, 312.5pg/mL. Buffer solution A is used as 0pg/mL.

(B)Sample volume 50μL (non- plasma and non-serum)

Reconstitute the Rat Leptin standard(lyophilized/20ng/vial) with 1mL of buffer solution B and 0.5ml of the reconstituted standard solution is diluted with 1.5 mL of buffer solution B which affords 5,000pg/mL standard solution. Then, 0.5ml of the standard solution is diluted with 0.5 mL of buffer solution B, that yields 2,500pg/mL standard solution. Repeat the dilution to make each standard of 1,250, 625, 312.5, 156.2, 78.1pg/mL. Buffer solution B is used as 0pg/mL.

2) Preparation of substrate solution:

Resolve an OPD tablet with 11 mL of substrate buffer. It should be prepared immediately before use.

3) Preparation of washing solution:
Dilute 50 mL of washing solution (concentrated) to 1000 mL with distilled or deionized water.

4) Other reagents are ready for use.

< Procedure >

1. Warm up the reagents and samples to room temperature before beginning the test.

2. Filling of standard solution and samples
(A) Sample volume 20μL(plasma and serum)
Fill 50μL of buffered solution A into wells first, then introduce 20μL each of standard solutions (0, 312.5, 625, 1,250, 2,500, 5,000, 10,000, 20,000pg/mL) or samples, then add 50μL of HRP labeled antibody. Total 120μL volume introduce into the wells.
(B) Sample volume 50μL (non-plasma and non-serum)
Fill 50μL each of standard solutions (0, 78.1, 156.2, 312.5, 625, 1,250, 2,500, 5,000pg/mL) or samples, then add 50μL of HRP labeled antibody. Total 100μL volume introduce into the wells.

3. Cover the plate with adhesive foil and incubate it at room temperature (20 ~ 30°C) for 5 hours. During the incubation, the plate should be rotated with a plate rotator.

4. Take off the adhesive foil, aspirate the solution in the wells and wash the wells three times with approximately 0.35 mL/well of washing solution.

5. Pipette 100μL of substrate solution into the wells, cover the plate with adhesive foil and incubate it for 10 minutes at room temperature.

6. Add 100μL of stopping solution into the wells to stop reaction.

7. Read the optical absorbance of the wells at 490nm.

8. Calculate mean absorbance values of wells containing standards and plot a standard curve on logarithmic graph paper (abscissa: concentration of standard; ordinate: absorbance value.).

9. Use the standard curve to read rat Leptin concentrations in samples from the corresponding absorbance values.

Ⅴ. Notes

1. Plasma or serum samples must be used as soon as possible after collection. If the samples are to be tested at a later time, they should be divided into test tubes in small amount and frozen at or below – 30°C. Avoid repeated freezing and thawing of plasma or serum samples.

2. Rat Leptin standard and substrate solution should be prepared immediately before use in assay using clean test tubes or vessels. Diluted washing solution is stable for 6 months at 2 to 8°C.

3. During storage of washing solution (concentrated) at 2 to 8°C, precipitates may be observed, however they will be dissolved when diluted.

4. As pipetting operations may affect with the precision of the assay, pipette precisely standard solutions or samples into each well of plate. And use new tip for each sample to avoid cross contamination.

5. When sample value exceeds 20,000pg/mL, it needs to be diluted with buffer solution A within the assay range.

6. During incubation except color reaction, the test plate should be rotated gently by plate rotator to promote immunoreaction.

7. During continuous rotation of test plate, the plate rotator may be heated up. It is recommended to place styrene form or plywood between the plate and the rotator.

8. Read optical absorbance of reaction solution in wells as soon as possible after stopping the color reaction.

9. Perform all the determination in duplicate.

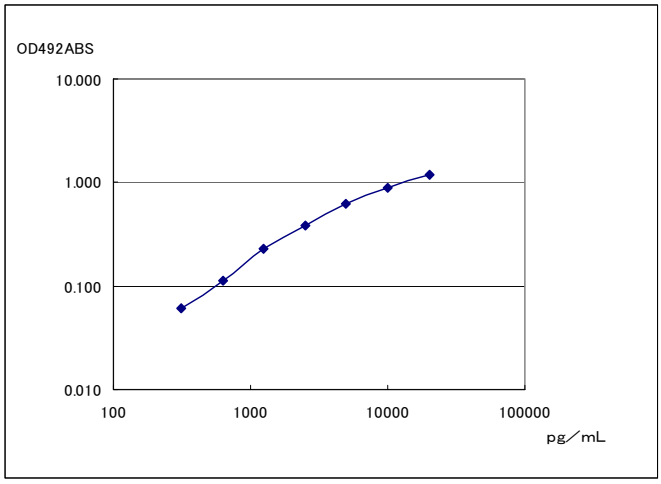
10. To quantitate accurately, always run a standard curve when testing samples.

11. Protect reagents from strong light (e.g. direct sunlight) during storage and assay.

12. Satisfactory performance of the test is guaranteed only when reagents are used from combination pack with identical lot number.

VI. Performance Characteristics

Typical standard curve



Analytical recovery A (Serum sample)

Rat Leptin added ng/mL	Observed ng/mL	Expected ng/mL	Recovery %
0	0	–	–
625	678	625	108.5
2,500	2,571	2,500	102.8
10,000	8,795	10.000	87.9

Analytical recovery B (culture supernatant sample)

Rat Leptin added ng/mL	Observed ng/mL	Expected ng/mL	Recovery %
0	0	–	–
156	169	156	108.0
625	668	625	106.9
2,500	2,482	2.500	99.3

Precision and reproducibility

- Intra-assay CV(%) 3.9 ~ 4.5
- Inter-assay CV(%) 6.2 ~ 9.5

Assay range

78.1 – 5,000pg/mL (non-plasma & non-serum sample)

312.5 – 20,000pg/mL (plasma & serum sample)

Ⅶ. Stability and Storage

< Storage > Store all of the components at 2 to 8°C.

< Shelf life > 8 month from the date of manufacturing
The expiry date is described on the label of kit.

< Package > For 96 tests per 1 kit including standards

Ⅷ. References

1. Zhang, Y. et al. (1994): Positional cloning of mouse obese gene and its human homologue. Nature **372:** 425.

2. Pelleymounter, M. A. et al. (1995): Effects of the obese gene product on body weight regulation in ob/ob mice. Science **299:** 540.

3. Funahashi, T. et al. (1995): Enhanced expression of rat obese (ob) gene in adipose tissues of ventromedial hypothalamus(VMH) – lesioned rats. Biochem. Biophys. Res. Commun. **211:** 469.

4. McGregor, G. P. et al. (1996): Radioimmunological measurement of leptin in plasma of obese and diabetis human subjects. Endocrinology **137:** 1501.

5. Sainsburry, A. et al. (1996): Intracerebroventricular administration of neuropeptide Y to normal rats increase obese gene expression in white adipose tissues. Diabetologia **39:** 469.

6. Hosoda, H. et al. (1996): Development of radioimmunoassay for human leptin. Biochem. Biophys. Res. Commun. **211:** 469.