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Rat Leptin ELISA Kit Instructions

For the quantitative determination of leptin
in rat serum or plasma and cell culture media

Catalog #90040
96 Assays

For research use only. Not for use in diagnostic procedures.

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A. Intended Use

The Rat Leptin ELISA kit is for the quantitative determination of leptin in rat serum, plasma, and fluid. Please read the complete kit insert before performing this assay. The kit is for *RESEARCH USE ONLY*. It is not intended for use in clinical or diagnostic procedures or for internal or external use in humans or animals.

B. Introduction

Obesity is a significant contributing factor in various adult diseases such as diabetes, cardiac disease, etc. This fact, combined with the increasing prevalence of obesity in the human population, has resulted in increased research on the underlying impact and cause of obesity.

In 1994, leptin, *obese* gene product, was identified from the investigation of ob/ob mouse. Leptin is a protein of about 16 kDa, which is expressed in adipose tissue, and promotes weight loss by suppressing appetite and stimulating metabolism. As a result, the accurate measurement of leptin in experimental animals is becoming increasingly important as obesity research intensifies.

The kit is a simple, precise, and sensitive ELISA sandwich assay for rat leptin.

Sensitivity range of the assay: 0.2 to 12.8 ng/mL using 5µL sample.

C. Principles of the Assay

1. First reaction

Rat leptin in the sample is simultaneously bound to the rabbit anti-leptin antibody coated on the microplate well and the anti-leptin IgG of the guinea pig anti-serum added to each well.

2. Washing

Unbound material is removed by washing.

3. Second reaction

Horse radish peroxidase (POD)-conjugated anti-guinea pig IgG antibody is then bound to the guinea pig anti-leptin IgG of the complex immobilized to the microplate well.

4. Washing

Excess POD-conjugated antibody is removed by washing.

5. Enzyme reaction

The bound POD conjugated antibody in the microplate well is detected by the addition of the 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution.

6. Measurement of absorbance

7. Evaluation of results

The leptin concentration is determined via interpolation using the standard curve generated by plotting absorbance versus the corresponding concentration of rat leptin standard.

D. Kit Storage

1. Upon receipt of the Rat Leptin ELISA kit, store it at 2-8°C and avoid light exposure (do not freeze the kit or hold it at temperatures above 25°C).

2. The kit should not be used after the expiration date.

E. Assay Materials

E.1. Materials supplied

TABLE 1 Contents of the kit

Mark	Description	Amount
A	Antibody-coated Microplate (One pack contains 6×8 well modules, *i.e.*, 48 wells / pack)	2 packs
B	Rat Leptin Standard, Lyophilized	2.56 ng/vial (for 100 µL)
C	Sample Diluent	1 bottle (50 mL)
D	Guinea Pig Anti-Leptin Serum	1 bottle (6 mL)
E	Anti-Guinea Pig IgG Enzyme Conjugate Stock Solution	1 bottle (8.4 mL)
F	Enzyme Conjugate Diluent	1 bottle (3.6 mL)
G	Enzyme Substrate (TMB) Solution	1 bottle (13 mL)
H	Enzyme Reaction Stop Solution (1 N Sulfuric Acid)	1 bottle (13 mL)
I	Wash Buffer Stock Solution (20X Concentrate)	1 bottle (50 mL)
	Frame for affixing the microplate well module	1 piece
	Plastic microplate cover	1 piece

E.2. Materials required but not provided

Micropipettes and disposable tips
Volumetric flasks
Distilled or deionized water
Polypropylene microtubes
Test tube racks
Vortex mixer
Aspirator for washing procedure
Microplate reader (capable of measuring A_{450} and A_{630} values)

F. Reagent Precautions

1. Avoid direct contact with the Enzyme Substrate Solution (marked "G") and the Enzyme Reaction Stop Solution (marked "H"). In case of contact, immediately flush eyes or skin with plenty of water and get medical advice.
2. Do not allow the Enzyme Substrate Solution (marked "G") to contact any metal.
3. The color and clarity of the Enzyme Conjugate Diluent (marked "F") may vary from lot-to-lot, but those variations will not have any influence on kit performances or results.
4. Only appropriately-trained personnel should use the kit. Laboratory personnel should wear suitable protective clothing. All chemicals should be considered potentially hazardous.

G. Maximizing Kit Performance

1. Given the small sample volumes required (5 µL), pipetting should be done as carefully as possible. A high quality 10 µL or better precision pipette should be used for such volumes. Drops of liquid adhering to the outside of the pipette tips should be removed by wiping to ensure the highest degree of accuracy.
2. In order to prevent the microplate wells from drying, samples and reagents should be dispensed quickly into the wells. In no case should 10 minutes be exceeded per plate per pipetting step.
3. The wash procedure should be done thoroughly in order to minimize background readings.
4. Each standard and sample should be assayed in duplicate.
5. The same sequence of pipetting and other operations should be maintained in all procedures.
6. Do not mix reagents that have different lot numbers.

H. Preparation of Rat Plasma and Serum

Plasma: Collect blood into a tube containing an anticoagulant such as heparin (final concentration: 1 unit/mL), EDTA (final concentration: 0.1%), or sodium citrate (final concentration: 0.76%), and centrifuge for 20 min at 2,000 x *g.*

Serum: Collect blood, allow to clot, and centrifuge for 20 min at 2,000 x *g*.

***Note*:** *Be sure to avoid hemolysis during preparation. Do not use turbid serum or plasma samples. Turbid serum or plasma should be centrifuged to produce a clear solution. Samples which need to be diluted must be diluted using the Sample Diluent (marked "C").*

I. Assay (0.2 – 12.8 ng/mL)

I.1. Preparation of reagents

Prior to use, all reagents should be brought to room temperature (18-25°C), and should be stored at 2-8°C immediately after use. Before use, mix the reagents thoroughly by gentle agitation or swirling.

1. Antibody-coated microplate
   Remove the "Antibody-coated Microplate" (marked "A") from the sealed foil pouch after the pouch has been equilibrated to room temperature.
   ***Note**: The microplate must be used the same day as the pouch is opened.*
2. Rat leptin stock solution
   Reconstitute the "Rat Leptin Standard, Lyophilized" (marked "B") by careful addition of 100 µL of sample diluent to the vial. Invert the vial gently until the contents are completely dissolved. This stock solution contains 25.6 ng/mL of rat leptin. The reconstituted rat leptin stock solution is stable for one week at 2-8°C.
3. Sample diluent
   The "Sample Diluent" (marked "C") is provided as a ready-to-use preparation. Once the bottle is opened, the sample diluent is stable for one week at 2-8°C.
4. Guinea pig anti-leptin serum
   The "Guinea Pig Anti-Leptin Serum" (marked "D") is provided as a ready-to-use preparation. Once the bottle is opened, the guinea pig anti-leptin serum is stable for one week at 2-8°C.
5. Anti-guinea pig IgG enzyme conjugate
   Mix the bottle of "Anti-Guinea Pig IgG Enzyme Conjugate Stock Solution" (marked "E") with the bottle of "Enzyme Conjugate Diluent" (marked "F"). Mix completely to ensure a homogeneous solution. Avoid foaming during mixing. The anti-guinea pig IgG enzyme conjugate is stable for one week at 2-8°C.
   ***Note**: The anti-guinea pig IgG enzyme conjugate is not needed till the second day of the assay. Color and clarity of the Conjugate may vary from lot-to lot.*
6. Enzyme substrate solution
   The "Enzyme Substrate Solution" (marked "G") is provided as a ready-to-use preparation. Once the bottle is opened, the

enzyme substrate solution is stable for one week at 2-8°C.

***Note**: Avoid exposure of the enzyme substrate solution to light.*

7. Enzyme reaction stop solution (1 N sulfuric acid)

   The "Enzyme Reaction Stop Solution" (marked "H") is provided as a ready-to-use preparation.

8. Wash buffer

   The "Wash Buffer Stock Solution" (marked "I") should be brought to 1 L with distilled or deionized water in a volumetric flask. Mix the solution well before use. The wash buffer is stable for one week at 2-8°C.

I.2. Preparation of working rat leptin standards

1. Pipette 50 µL of sample diluent (marked "C") and 50 µL of rat leptin stock solution (25.6 ng/mL) into a polypropylene microtube labeled 12.8 ng/mL, and mix thoroughly.
2. Dispense 50 µL of sample diluent into six polypropylene microtubes labeled 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng/mL, respectively.
3. Dispense 50 µL of the 12.8 ng/mL standard into the 6.4 ng/mL microtube, and mix thoroughly.
4. Dispense 50 µL of the 6.4 ng/mL standard into the 3.2 ng/mL microtube, and mix thoroughly.
5. Repeat this dilution scheme using the remaining microtubes.
6. Dispense 50 µL of sample diluent into one polypropylene microtube labeled 0 ng/mL.

***Note**: The working leptin standards should be prepared shortly before use and discarded after use. Prepare working leptin standards using polypropylene microtubes because polypropylene exhibits minimal adsorption of leptin.*

TABLE 2 Preparation of working rat leptin standards

	Rat leptin concentration (ng/mL)							
	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0
RLSS*	50µL							
SD**	50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL
		50µL	50µL	50µL	50µL	50µL	50µL	
Total	100µL	100µL	100µL	100µL	100µL	100µL	100µL	50µL

RLSS*: Rat Leptin Stock Solution (25.6 ng/mL)
SD**: Sample Diluent

I.3. Assay Procedure

First reaction:

1. Remove the antibody-coated microplate modules (marked “A”) from the sealed foil pouch after the pouch has been equilibrated to room temperature. Affix the microplate modules to the supporting frame.
2. Wash the plate two times using 300 µL of wash buffer per well. After each wash, remove any remaining solution by inverting and tapping the plate firmly on a clean paper towel.
3. In each well, dispense 45 µL of sample diluent (marked “C”).
4. In each well, dispense 50 µL of guinea pig anti-rat leptin serum (marked “D”).
5. Pipette 5 µL samples (or 0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 ng/mL working rat leptin standards) into the wells.

   ***Note**: Each standard and sample should be assayed in duplicate. It is also recommended that a 10 µL or better precision pipette be used when dispensing small volumes (5 µL).*
6. Cover the microplate with the plastic microplate cover and incubate overnight (16 – 20 hours) at 4°C.

Second reaction:

7. Aspirate well contents and wash five times using 300 µL of wash buffer per well. After each wash, remove any remaining solution by inverting and tapping the plate firmly on a clean paper towel.
8. Dispense 100 µL per well of anti-guinea pig IgG enzyme conjugate.
9. Cover the microplate with the plastic microplate cover and incubate for 3 hours at 4°C.

Third reaction:

10. Aspirate well contents and wash seven times using 300 µL of wash buffer per well. After each wash, remove any remaining solution by inverting and tapping the plate firmly on a clean paper towel.
11. Immediately dispense 100 µL per well of enzyme substrate solution (marked “G”) and react for 30 minutes at room temperature. During the enzyme reaction, avoid exposing the microplate to light.

    ***Note**: Do not cover the microplate with aluminum foil.*
12. Stop the enzyme reaction by adding 100 µL per well of enzyme reaction stop solution (marked “H”).
13. Measure absorbance within 30 minutes using a plate reader. (Measure A_{450} values and subtract A_{630} values).

I.4. Determining the leptin concentration

1. Determine the mean absorbance for each set of duplicate standards or samples.

   ***Note**: If individual absorbance values differ from the mean by greater than 20%, performing the assay again is recommended. The mean absorbance of the 0 ng/mL standard should be less than 0.1.*

2. Using semi-log graph paper, construct the leptin standard curve by plotting the mean absorbance value for each standard on the Y axis versus the corresponding standard rat leptin concentration on the X axis. Figure 1 is an example of a typical standard curve generated by the assay.

   ***Note**: A standard curve should be plotted every time the assay is performed.*

3. Rat leptin concentrations in the samples are interpolated using the standard curve and mean absorbance values for each sample.

   ***Note**: Samples with a high leptin concentration (12.8 ng/mL or higher) should be diluted with the sample diluent and rerun.*

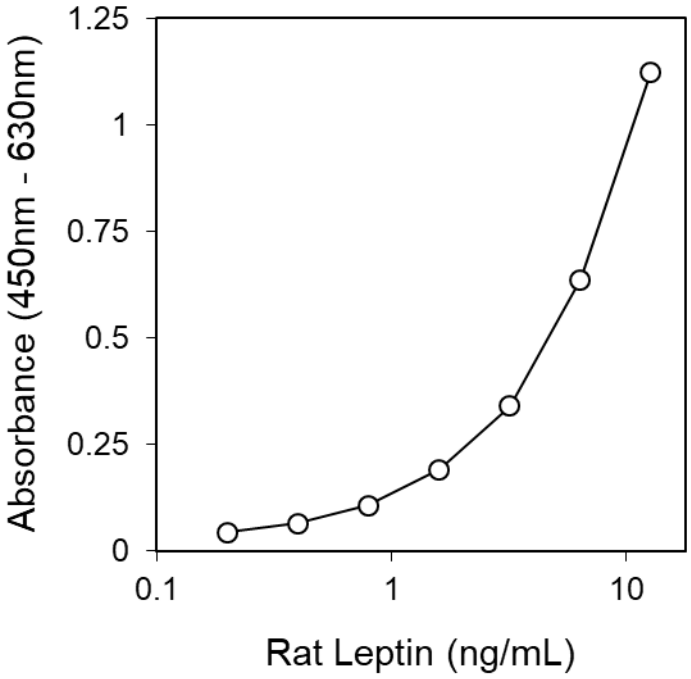

Figure 1: Typical rat leptin standard curve

J.Appendix

J.1. Performance characteristics

1. Precision: The intra-assay precision: C.V. ≤ 10%
   The inter-assay precision: C.V. ≤ 10%

2. Recovery: When rat leptin was spiked in rat serum sample, the recovery was 100% ± 20 %.

J.2. Rat leptin recovery test

To determine the recovery of rat leptin, a minimum of three sample assays should be performed as illustrated in Table 3 (A, C, D or B, C, E).

TABLE 3 Example worksheet using a 5 µL sample

Unknown sample (µL)	Rat leptin standard (µL)	Sample diluent (µL)	Guinea pig anti-leptin serum (µL)	Total volume (µL)	Actual value (ng/mL)
0	5 (1.6 ng/ml)	45	50	100	1.46 **A**
0	5 (3.2 ng/ml)	45	50	100	3.09 **B**
5	0 (0 ng/ml)	45	50	100	2.80 **C**
5	5 (1.6 ng/ml)	40	50	100	3.53 **D**
5	5 (3.2 ng/ml)	40	50	100	5.19 **E**

Once the assays have been performed, use either of the calculations below to determine the leptin recovery:

Calculation 1

$$\text{Recovery (\%)} = \frac{\textbf{D}\ (3.53\ \text{ng/mL})}{\textbf{A}\ (1.46\ \text{ng/mL}) + \textbf{C}\ (2.80\ \text{ng/mL})} \times 100 = \textbf{82.9}$$

Calculation 2

$$\text{Recovery (\%)} = \frac{\textbf{E}\ (5.19\ \text{ng/mL})}{\textbf{B}\ (3.09\ \text{ng/mL}) + \textbf{C}\ (2.80\ \text{ng/mL})} \times 100 = \textbf{88.1}$$

J.3. Summary of reagent preparation

TABLE 4 – Summary of reagent preparation

Reagent	Preparation Procedure
A: Antibody-coated Microplate	Ready to use
B: Rat Leptin Standard, Lyophilized	Dilute with 100 µL of Sample Diluent (marked "**C**")
C: Sample Diluent	Ready to use
D: Guinea Pig Anti-Leptin Serum	Ready to use
E: Anti-Guinea Pig IgG Enzyme Conjugate Stock Solution	Mix the bottle of **E** with the bottle of **F** and mix completely**
F: Enzyme Conjugate Diluent	
G: Enzyme Substrate (TMB) Solution	Ready to use
H: Enzyme Reaction Stop Solution (1 N Sulfuric Acid)	Ready to use
I: Wash Buffer Stock Solution (20X Concentrate)	Bring contents of the bottle to 1 L with water*

***Note**: All reagents should be brought to room temperature (18-25°C) prior to use.*

* Distilled or deionized water.

** Not needed till the second day of the assay.

J.4. Summary of Rat Leptin ELISA kit assay

Affix the Antibody-coated Microplate (marked "A") to the frame

↓

Wash each well two times with wash buffer*

↓

Dispense 45 µL of Sample Diluent (marked "C") per well

↓

Dispense 50 µL of Guinea Pig Anti-Leptin Serum (marked "D") per well

↓

Pipette 5 µL of the sample (or working rat leptin standard) per well

↓

Incubate the microplate overnight (16 -20 hours) at 4°C

↓

Wash each well five times with wash buffer*

↓

Dispense 100 µL of anti-guinea pig IgG enzyme conjugate per well

↓

Incubate the microplate for 3 hours at 4°C

↓

Wash each well seven times with wash buffer*

↓

Dispense 100 µL of Enzyme Substrate Solution (marked "G") per well

↓

Incubate microplate for 30 min at room temperature while avoiding exposure to light.

↓

Stop the enzyme reaction by adding 100 µL of Enzyme Reaction Stop Solution (marked "H") per well

↓

Measure A_{450} and subtract A_{630} values within 30 min

↓

Calculate leptin concentrations using the standard curve

* Each well should be washed with 300 µL of wash buffer. Aspirate the wells completely so all excess solution is removed.

Warranty

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