> Leptin (rat), ELISA Kit

Catalog # ADI-900-015A

96 Well Enzyme Immunoassay Kit

For use with serum, plasma and culture supernates

Check our website for additional protocols, technical notes and FAQs.

For proper performance, use the insert provided with each individual kit received.

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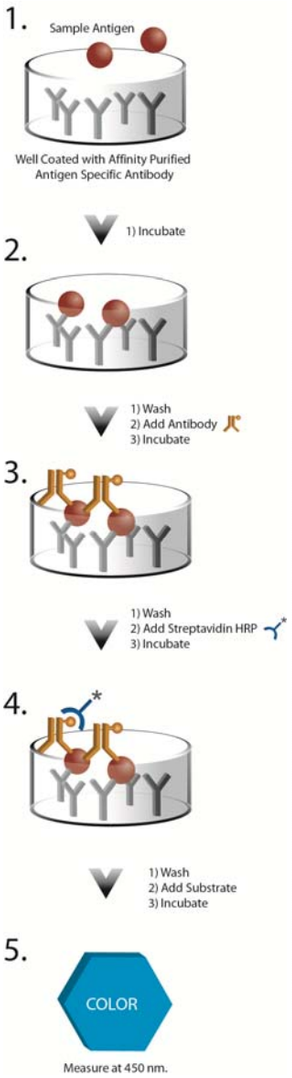


Introduction

The Leptin (rat) enzyme-linked immunosorbent assay (ELISA) kit is a complete kit for the quantitative determination of leptin in culture supernates, serum and plasma of rat origin. Please read the entire kit insert before performing this assay.

The adipokine leptin is a hormone secreted predominantly by adipose tissue that signals through leptin receptors in the hypothalamus to decrease appetite and increase energy expenditure[1,2]. Binding of leptin to the long-form of the leptin receptor in the hypothalamus reduces neuropeptide Y (NPY) and agouti-related protein (AgRP) activity, while stimulating anorexigenic pro-opiomelanocortin (POMC) neuron activity to reduce appetite[3,4]. In peripheral tissues, leptin antagonizes insulin signaling, increases fatty acid oxidation, decreases insulin production in pancreatic ß cells, and promotes fertility. Its expression in adipocytes can be regulated in a paracrine fashion by other adipokines such as IL-6 (stimulation) and TNF-α (inhibition)[5].

Leptin deficient mice display severe insulin resistance, obesity, and decreased fertility, all of which are reversible by administration of exogenous leptin[6,7]. In contrast, elevated circulating levels of leptin are associated with increased obesity, indicative of an acclimated state of leptin resistance which is not well understood. Evidence also links leptin to cardiovascular disease, as it mediates numerous pro-inflammatory and pro-atherogenic effects such as increased endothelin-1 (ET-1) expression, accumulation of reactive oxygen species (ROS), secretion of monocyte chemoattractant protein-1 (MCP-1), and increased sympathetic vascular tone and blood pressure[5].

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

Principle

1. Samples and standards are added to wells coated with a polyclonal antibody specific for leptin. The plate is then incubated.
2. The plate is washed, leaving only bound leptin on the plate. A solution of biotinylated antibody specific for leptin is then added. This binds the leptin captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution of streptavidin conjugated to horseradish peroxidase is added to each well, binding to the biotinylated antibody. The plate is again incubated.
4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of leptin in the sample.

Materials Supplied

1. Assay Buffer 28

   100 mL, Catalog No. 80-1797

   Phosphate buffered saline containing BSA and detergent

2. Recombinant Leptin (rat) Standard

   Catalog No. 80-1790

   Two vials containing 6400 pg of recombinant rat leptin

The standard should be handled with care due to the known and unknown effects of the antigen.

3. Leptin Clear Microtiter Plate

   One plate of 96 wells, Catalog No. 80-1793

   A clear plate of break-apart strips coated with a goat polyclonal antibody specific for leptin

4. Leptin EIA Antibody

   Catalog No. 80-1795

   One vial containing biotinylated antibody to leptin

Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.

5. Leptin (rat) EIA Conjugate

   10 mL, Catalog No. 80-1791

   A blue solution of streptavidin conjugated to horseradish peroxidase

6. Wash Buffer Concentrate

   100 mL, Catalog No. 80-1287

   Tris buffered saline containing detergents

Protect substrate from prolonged exposure to light.

7. TMB Substrate

   10 mL, Catalog No. 80-0350

   A solution of 3,3’5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide

Stop solution is caustic. Keep tightly capped.

8. Stop Solution 2

   10 mL, Catalog No. 80-0377

   A 1N solution of hydrochloric acid in water

9. Leptin (rat) Assay Layout Sheet

   1 each, Catalog No. 30-0252

10. Plate Sealer

    2 each, Catalog No., 30-0012

All reagents should be stored at 4°C.

Storage

All components of this kit are stable at 4°C until the kit's expiration date.

Materials Needed but Not Supplied

1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater pipet for dispensing 100 µL
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. A microplate shaker
7. Lint-free paper for blotting
8. Microplate reader capable of reading at 450 nm
9. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit

Reagent Preparation

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Glass or plastic tubes may be used for standard preparation.

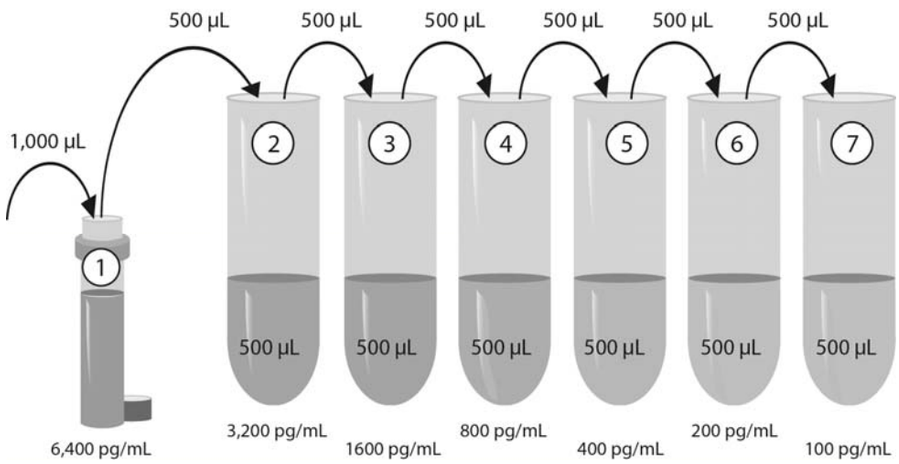
1. **Wash Buffer**

   Prepare the wash buffer by diluting 50 mL of the supplied Wash Buffer Concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit's expiration, or for 3 months, whichever is earlier.

2. **Leptin ELISA Antibody**

   Reconstitute the vial with 1 mL Assay Buffer 28 and gently vortex. Wait 5 minutes and vortex again. To make the working stock, mix 1 part concentrate with 10 parts assay buffer. For example, if making 5.5 mL working stock, add 500 µL concentrate to 5 mL assay buffer. Antibody concentrate can be stored at 4°C for up to 3 months or aliquoted and stored at -20°C for up to 1 year. Do not store the antibody working stock.

3. **Preparation of Leptin Standard Curve**



   Reconstitute the 6400 pg lyophilized standard vial with 1 mL Assay Buffer 28 and gently vortex. Wait 5 minutes and vortex again. This solution contains 6400 pg/mL and is tube #1. Label six tubes #2 through #7. Pipet 500 µL of the assay buffer into tubes # 2 through #7. Pipet 500 µL of the 6400 pg/mL standard to tube #2. Vortex. Add 500 µL of tube #2 into tube #3 and vortex thoroughly. Continue this for tubes #3 through #7.

   Diluted standards should be used within 1 hour of preparation.

   The concentrations of the standards are labeled above.

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

Sample Handling

Rat culture supernates, serum, heparin plasma, and Na EDTA plasma are suitable for use in this assay; other sample matrices have not been validated. Prior to assay, samples should be slowly brought to 4˚C and centrifuged to remove debris. Due to differences in samples, users must determine the optimal sample dilution for their particular experiments.

A minimum 1:4 dilution in the assay buffer is required for most serum and plasma samples. Most culture supernates without serum supplementation require a minimum 1:2 dilution in the assay buffer. No dilution is required for most culture supernates supplemented with 10% fetal bovine serum (FBS). These are the minimum dilutions required to eliminate matrix interference in the assay. Below are examples of the linearity experiments performed to determine the optimal sample dilution for these samples.

Changes in binding associated with different media may be corrected by using the same non-conditioned media as the standard diluent.

Dilutional Linearity

The minimum required dilution for several common samples was determined by serially diluting samples into the assay buffer and identifying the dilution at which linearity is observed. Non-conditioned Dulbecco's Modified Eagle's Medium (DMEM) with or without 10% fetal bovine serum (FBS) was spiked with recombinant leptin and diluted in the assay buffer. The assay buffer was spiked to the same concentration and used as a control to determine linearity of the culture medium. Pools of natural rat serum, heparin plasma, and Na EDTA plasma were also diluted in the assay buffer to produce values within the dynamic range of the assay.

	Average % of Expected				
Dilution	**Culture Media (DMEM)**	**Culture Media (DMEM + 10% FBS)**	**Serum**	**Heparin Plasma**	**Na EDTA Plasma**
Neat	23	85	73	52	57
1:2	103	106	84	81	72
1:4	104	99	94	96	86
1:8	---	---	97	104	98
1:16	---	---	98	110	101

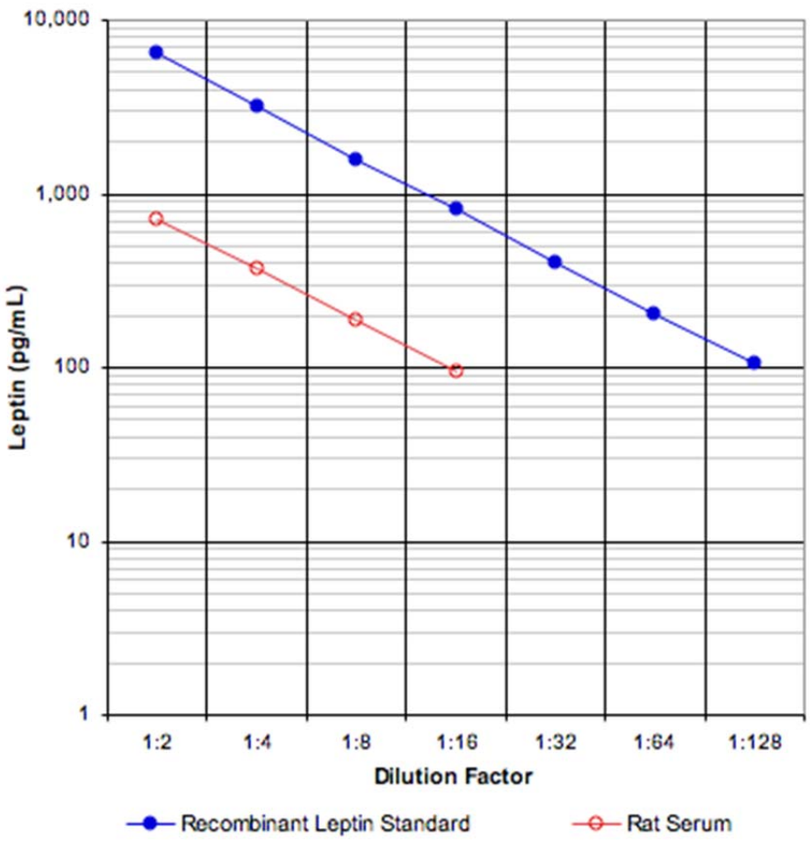
Spike and Recovery

After diluting each sample matrix to its minimum required dilution, recombinant rat leptin was spiked at high, medium, and low concentrations. The recovery of the standard in spiked samples was compared to the recovery of identical spikes in the assay buffer. The mean and range of percent recovery at the three concentrations are indicated below for each matrix.

Sample Matrix (# of samples)	Minimum Required Dilution	Spike Concentration (pg/mL)	Recovery of Spike (Range)
DMEM (n = 1)	1:2	2700	106% (n/a)
		900	111% (n/a)
		300	103% (n/a)
DMEM + 10% FBS (n = 1)	Neat	2700	86% (n/a)
		900	95% (n/a)
		300	86% (n/a)
Serum (n = 8)	1:4	2700	79% (68-95%)
		900	74% (67-88%)
		300	85% (61-107%)
Heparin Plasma (n = 5)	1:4	2700	83% (80-86%)
		900	83% (69-96%)
		300	96% (82-116%)
Na EDTA Plasma (n = 6)	1:4	2700	99% (88-114%)
		900	86% (66-98%)
		300	97% (80-116%)

Parallelism

A parallelism experiment was carried out to determine if the recombinant leptin standard accurately determines leptin concentrations in biological matrices. To assess parallelism, values for rat serum were obtained from a standard curve using four parameter logistic curve fitting. The observed concentration was plotted against the dilution factor. Parallelism of the curves demonstrates that the antibody binding characteristics are similar enough to allow the accurate determination of analyte levels in diluted samples.



Serum and Plasma Preparation

1. Collect whole blood in appropriate tubes for either serum or plasma.
2. Allow serum to clot for 30 minutes.
3. Centrifuge at 1000 x g for 15 minutes at 4°C.
4. Place supernatants in a clean tube.
5. The supernatant may be aliquoted and stored at or below -20°C, or used immediately in the assay.
6. Avoid repeated freeze-thaw cycles.

Assay Procedure

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4˚C.

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addition of the antibody, conjugate and substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

1. Pipet 100 µL of the assay buffer into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #7 to the bottoms of the appropriate wells.
3. Pipet 100 µL of the samples to the bottoms of the appropriate wells.
4. Seal the plate and incubate for 1 hour shaking* at room temperature.
5. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100 µL of antibody into each well except the blank.
7. Seal the plate and incubate for 1 hour shaking* at room temperature.
8. Wash as above (Step 5).
9. Pipet 100 µL of blue conjugate to each well except the blank.
10. Seal the plate and incubate for 30 minutes shaking* at room temperature.
11. Wash as above (Step 5).
12. Pipet 100 µL of substrate solution into each well.
13. Incubate for 30 minutes shaking* at room temperature.
14. Pipet 100 µL of stop solution into each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700 rpm.

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Calculation of Results

Several options are available for the calculation of the concentration of leptin in the samples. We recommend that the data be handled by an immunoassay software package utilizing a four parameter logistic curve fitting program. Such software is often supplied by plate reader manufacturers. If data reduction software is not readily available, the concentrations can be calculated as follows:

1. Calculate the average Net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

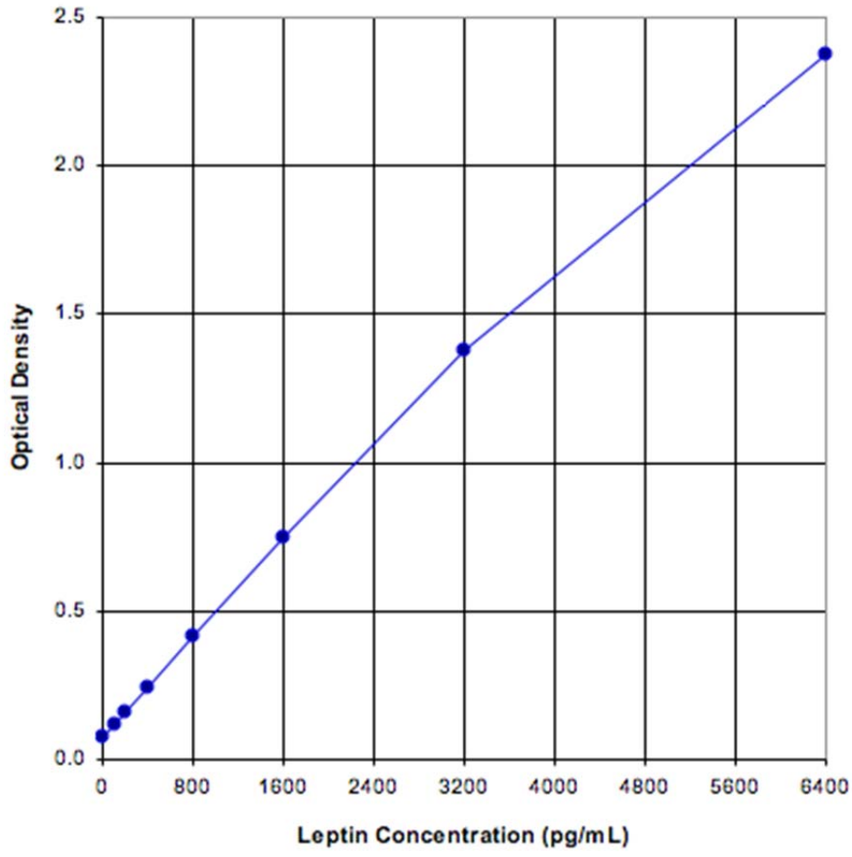
2. Using linear graph paper, plot the average Net OD for each standard versus leptin concentration in each standard. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Net OD	pGSK-3β (pg/mL)
S0	0.076	0
S1	2.374	6400
S2	1.381	3200
S3	0.749	1600
S4	0.414	800
S5	0.242	400
S6	0.156	200
S7	0.115	100



Performance Characteristics

Specificity

The cross reactivities of related compounds were determined by diluting cross reactants in the assay buffer at several concentrations. These cross reactants were then measured in the assay.

Compound	Cross Reactivity
Rat Leptin	100%
Mouse Leptin	318%
Human Leptin	0.5%

Sensitivity

The sensitivity or limit of detection of the assay is 67.2 pg/mL. The sensitivity was determined by interpolation at 2 standard deviations above the mean signal at background (0 pg/mL) using data from 9 standard curves.

Precision

Intra-assay precision was determined in by assaying 20 replicates of three buffer controls containing leptin in a single assay.

pg/mL	%CV
2070	2.5
906	7.1
241	8.0

Inter-assay precision was determined by measuring buffer controls of varying leptin concentrations in multiple assays over several days.

pg/mL	%CV
2078	4.1
1152	6.5
317	11

References

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