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TiterZyme® EIA

rat Leptin

Enzyme Immunometric Assay Kit

Catalog No. 900-015

96 Determination Kit

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Description

Assay Designs' rat Leptin TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of rat Leptin in biological fluids. Please read the complete kit insert before performing this assay. Assay Designs' kit uses a polyclonal antibody to Leptin immobilized on a microtiter plate to bind the Leptin in the standards or sample. A recombinant rat Leptin Standard is provided in the kit. After a short incubation the excess standard or sample is washed out and a polyclonal antibody to rat Leptin labeled with the enzyme Horseradish peroxidase is added. This labeled antibody binds to the Leptin captured on the plate. After a short incubation the excess labeled antibody is washed out and substrate is added. The substrate reacts with the labeled antibody bound to the rat Leptin captured on the plate. The color generated in a 30 minute incubation with the substrate is read at 450 nm, and is directly proportional to the concentration of rat Leptin in the sample. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

The obesity gene that encodes for Leptin was originally identified in 1994 by Freidman's group at Rockerfeller University[3]. Leptin is a 16,000 molecular weight protein produced by the *ob* gene. Adipocytes produce Leptin and release it into the bloodstream. As fat deposits grow, blood Leptin levels tend to increase[4]. Is has been suggested that Leptin acts as a lipostat, increasing as fat gets deposited into adipocytes[5-10]. It is also clear that the protein acts as a hormone instructing the brain to stop food consumption and to increase activity[5,11]. Leptin interacts and perhaps controls the levels of a potent 36 amino acid appetite stimulating neurotransmitter, neuropeptide Y (NPY)[9,12]. NPY production is suppressed in animals given Leptin. The protein has also been shown to signal and perhaps control the onset of puberty.

Precautions

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1. Stop Solution is a 1 normal (1N) sulfuric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles, such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however, it is possible that high levels of interfering substances may cause variation in assay results.
4. The rat Leptin Standard provided, Catalog No. 80-0172, should be handled with care because of the known and unknown effects of Leptin.

Materials Supplied

1. **rat Leptin Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0168**
   A strip microtiter plate coated with rabbit antibody specific to rat Leptin.
2. **rat Leptin Labeled Antibody Concentrate, 0.4 mL, Catalog No. 80-1356**
   Rabbit antibody to rat Leptin conjugated to Horseradish peroxidase.
3. **Assay Buffer, 30 mL, Catalog No. 80-0170**
   Phosphate buffered saline containing proteins and detergents.
4. **Labeled Antibody Diluent, 12 mL, Catalog No. 80-0182**
   Phosphate buffered saline containing proteins and detergents.
5. **Wash Buffer Concentrate, 50 mL, Catalog No. 80-0171**
   Phosphate buffered saline containing detergents.
6. **rat Leptin Standard, 2 each, Catalog No. 80-0172**
   Two vials containing 3,600 pg each of lyophilized recombinant rat Leptin.
7. **TMB Substrate, 15 mL, Catalog No. 80-1342**
   A solution of 3,3',5,5' tetramethylbenzidene (TMB) and hydrogen peroxide. Ready to use.
8. **Stop Solution, 10 mL, Catalog No. 80-0176**
   A 1N solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **rat Leptin Assay Layout Sheet, 1 each, Catalog No. 30-0040**
10. **Plate Sealer, 2 each, Catalog No. 30-0012**

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 100 µL and 1,000 µL.
3. Repeater pipet for dispensing 100 µL.
4. Disposable beaker for diluting buffer concentrates.
5. Graduated cylinders.
6. A 37°C incubator.
7. Microplate reader capable of reading at 450 nm, preferably with correction between 570 and 590 nm.
8. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' TiterZyme® EIA is compatible with rat Leptin samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

Culture fluids, serum or EDTA plasma are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can be read in the assay if diluted into Assay Buffer. Users should only use standard curves generated in Assay Buffer to calculate concentrations of rat Leptin.

Samples must be stored frozen to avoid loss of bioactive rat Leptin. If samples are to be run within 24 hours, they may be stored at 4°C, otherwise samples must be stored frozen at -70°C. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37°C incubator. Do not vortex or sharply agitate samples.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses plates with removable strips. Unused strips must be kept desiccated at 4°C in the sealed bag provided. The strips should be used in the frame provided.
8. **Prior to addition of standard, antibody, and substrate, ensure that there is no residual wash buffer in these wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare Wash Buffer by diluting 25 mL of the supplied concentrate with 975 mL of deionized water. This can be stored at 4°C until the kit expiration, or for 3 months, whichever is earlier.

2. rat Leptin Standard

Add 500 µL of deionized water to the rat Leptin Standard. Let it sit at room temperature for 5 minutes. Mix it gently. This solution contains 7,200 pg/mL rat Leptin.

Label seven 12 x 75 mm glass tubes #1 through #7. Pipet 220 µL of Assay Buffer into tubes #1 through #7. Add 220 µL of the 7,200 pg/mL standard to tube #1. Vortex. Add 220 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #7.

The concentration of rat Leptin in tubes #1 through #7 will be 3,600, 1,800, 900, 450, 225, 113, and 56 pg/mL respectively. See rat Leptin Assay Layout Sheet for dilution details. Store reconstituted standard at or below -20°C, avoid repeated freeze/thaws.

3. Labeled Antibody Conjugate

Prepare the Labeled Antibody **immediately before use**. Do not store prepared Labeled Antibody solution. For each strip used, mix 30µL of the Labeled Antibody Concentrate with 870µL of the Labeled Antibody Diluent.

Assay Procedure

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

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining strips with the desiccant back into the foil pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of Assay Buffer into the S0 (0 pg/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at 37°C for 1 hour.
7. Empty the contents of the wells and wash the plate by adding 400 µL of wash solution to every well. Repeat the wash 6 more times for a total of **7 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.
8. Pipet 100 µL of the prepared Labeled Antibody solution into each well, except the Blank.
9. Seal the plate and incubate at 37°C for 30 minutes.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 8 more times for a total of **9 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.
11. Add 100 µL of the TMB Substrate to each well.
12. Incubate for 30 minutes at room temperature in the dark.
13. Add 100 µL of Stop Solution to each well.
14. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of rat Leptin in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of rat Leptin can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound 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 rat Leptin concen-tration in each standard. Approximate a straight line through the points. The concentration of rat Leptin in the unknowns can be determined by interpolation.

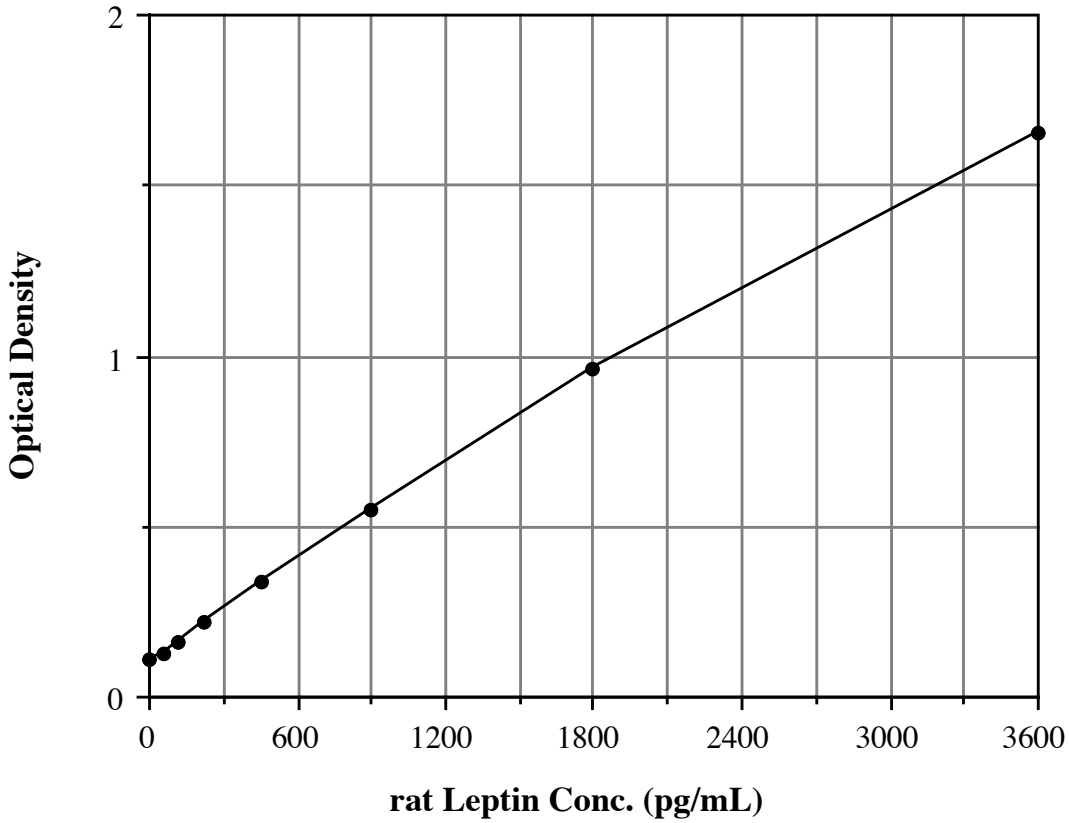
Typical Results

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

Sample	Average OD	Net OD	**rat Leptin (pg/mL)**
Blank	(0.042)		
0 standard	0.152	0.110	**0**
S1	1.697	1.655	**3600**
S2	1.005	0.963	**1800**
S3	0.591	0.549	**900**
S4	0.376	0.334	**450**
S5	0.258	0.216	**225**
S6	0.201	0.159	**113**
S7	0.169	0.127	**56**

Typical Standard Curve

The typical standard curve shown below **must not** be used to calculate rat Leptin concentrations; the user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[13].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run at 0 pg/mL rat Leptin, and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of rat Leptin measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Average Optical Density for the S0 =	0.099 ± 0.005
Average Optical Density for Standard #7 =	0.111 ± 0.010
Delta Optical Density (56-0 pg/mL) =	0.012
2 SD's of the 0 pg/mL Standard = 2 x 0.005 =	0.010

$$\text{Sensitivity} = \frac{0.010}{0.012} \times 56 \text{ pg/mL} = \textbf{46.7 pg/mL}$$

Linearity

A sample containing 1,895.54 pg/mL rat Leptin was diluted 7 times 1:2 into media, such as RPMI with 10% fetal bovine serum added and measured in the assay. The data was plotted graphically as actual rat Leptin concentration versus measured Leptin concentration.

The line obtained had a slope of 0.8422 and a correlation coefficient of 0.9995.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of rat Leptin and running these samples multiple times (n=32) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of rat Leptin in multiple assays (n=24).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of rat Leptin determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	rat Leptin (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	131	4.7	
Medium	578	4.1	
High	2,433	5.1	
Low	130		4.4
Medium	570		3.3
High	2,377		3.2

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant in Assay Buffer. These samples were then measured in the rat Leptin assay, and the measured rat Leptin concentration calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
rat Leptin	100%
mouse Leptin	17.9%
human Leptin	0.2%
rat GRO/CINC-1	<0.1%
rat GRO/CINC-2α	<0.1%
rat GRO/CINC-2β	<0.1%
rat GRO/CINC-3	<0.1%
rat MCP-1	<0.1%
rat Rantes	<0.1%
rat MIP-2	<0.1%
rat IL-1β	<0.1%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Rat Leptin concentrations were measured in tissue culture media, rat serum and rat EDTA plasma. Rat Leptin was spiked into the undiluted samples of these matrices which were then diluted with the kit Assay Buffer and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	91.4	≥1:2
Rat Serum	92.4	≥1:8
Rat EDTA plasma	97.8	≥1:8

*See Sample Handling Instructions on page 4 for details.

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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