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Modified Reagent Preparation and New Storage Conditions

TiterZyme® EIA

human Leptin

Enzyme Immunometric Assay Kit

Catalog No. 900-028

96 Determination Kit

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Description

Assay Designs' human Leptin TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of human Leptin in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to human Leptin immobilized on a microtiter plate to bind the human Leptin in the standards or sample. A recombinant human Leptin Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a rabbit polyclonal antibody to human Leptin labeled with the enzyme Horseradish peroxidase is added. This labeled antibody binds to the human 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 human Leptin captured on the plate. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of human Leptin in either standards or samples. 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 Rockefeller 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 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 human Leptin Standard provided, Catalog No. 80-0295, should be handled with care, because of the known and unknown effects of Leptin.

Materials Supplied

1. **human Leptin Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0293**
   A strip microtiter plate coated with rabbit antibody specific to human Leptin.
2. **human Leptin Labeled Antibody Concentrate, 0.4 mL, Catalog No. 80-1353**
   Rabbit antibody to human 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. **human Leptin Standard, 1 each, Catalog No. 80-0295**
   One vial containing 12,500 pg of recombinant human Leptin.
7. **TMB Substrate, 15 mL, Catalog No. 80-1342**
   A solution of 3.3',5.5' tetramethyl benzidine (TMB) and hydrogen peroxide. Ready to use.
8. **Stop Solution, 12 mL, Catalog No. 80-0176**
   A 1N solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **human Leptin Assay Layout Sheet, 1 each, Catalog No. 30-0084**
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. Disposable test tubes for dilution of samples and standards.
4. Repeater pipet for dispensing 100 µL.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A 37 °C incubator.
8. A 4 °C incubator.
9. Adsorbent paper for blotting.
10. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
11. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' TiterZyme® EIA is compatible with human 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.

In mammals, levels of human Leptin are in the range 1-200 ng/mL. Culture fluids, serum and 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 samples. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay if diluted into Assay Buffer. Users should only use standard curves generated in Assay Buffer to calculate concentrations of human Leptin.

Procedural Notes

1. Do not mix reagents from different lot numbers 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 standard 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 dessicated 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 date, or for 3 months, whichever is earlier.

2. human Leptin Standards

Add 500 µL of deionized water to the human Leptin Standard. Let it stand at room temperature for 5 minutes. Mix gently. This solution contains 25,000 pg/mL human 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 25,000 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 human Leptin in tubes #1 through #7 will be 12,500, 6,250, 3,125, 1,562, 781, 391 and 195 pg/mL respectively. See human Leptin Assay Layout Sheet for dilution details. Store the reconstituted standard at or below -20 °C, avoid repeated freeze/thaws.

3. Labeled Antibody Conjugate

Prepare labeled Antibody solution **immedately before use**. Do not store prepared labeled Antibody solution. For each strip used, mix 30 µL of labeled Antibody concentrate with 870 µL of 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 strips to be used and put any remaining strips back with the desiccant into the pouch and seal the ziploc. Store unused strips 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 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 into each well, except the Blank.

9. Seal the plate and incubate at 4 °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 human 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 human 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. Plot the Average Net OD for each standard versus human Leptin concentration in each standard.

3. Using linear graph paper, plot the Average OD for each standard versus human Leptin concentration in each standard. Approximate a straight line through the points. The concentration of human Leptin in the unknowns can be determined by interpolation.

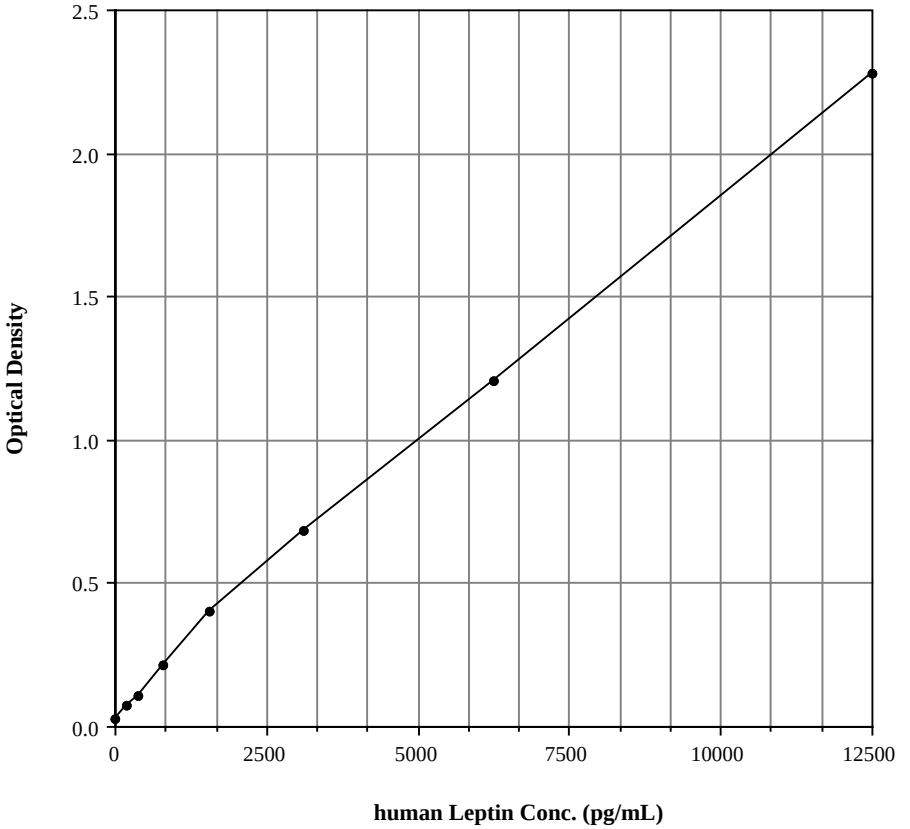
Typical Results

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

Sample	Average OD	Net OD	**human Leptin (pg/mL)**
Blank	(0.041)		
S0	0.070	0.029	**0**
S1	2.320	2.279	**12,500**
S2	1.249	1.208	**6,250**
S3	0.722	0.681	**3,125**
S4	0.443	0.402	**1,562**
S5	0.255	0.214	**781**
S6	0.148	0.107	**391**
S7	0.113	0.072	**195**

Typical Standard Curve

The typical standard curve shown below **must not** be used to calculate human Leptin concentrations; each 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 eight (8) wells run at 0 pg/mL Standard, and comparing to the average optical density for eight (8) wells run with Standard #7. The detection limit was determined as the concentration of human Leptin measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Average Optical Density for the S0 =	0.094 ± 0.004 (4.2%)
Average Optical Density for Standard #7 =	0.155 ± 0.002 (1.4%)
Delta Optical Density (195-0 pg/mL) =	0.061
2 SD's of the 0 pg/mL Standard = 2 x 0.004 =	0.008

Sensitivity = $\frac{0.008}{0.061}$ x 195 pg/mL = **25.5 pg/mL**

Linearity

A sample containing 12,430.253 pg/mL human Leptin was serially diluted 5 times 1:2 into the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual human Leptin concentration versus measured human Leptin concentration.

The line obtained has a slope of 1.022 with a correlation coefficient of 0.998.

Precision

Intra-assay precision was determined by taking samples containing low and high concentrations of human Leptin and running these samples multiple times (n=40) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of human Leptin in multiple assays (n=24).

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

	human Leptin (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	366.97	9.4	
Medium	1,727.42	12.9	
High	6,146.99	7.8	
Low	349.45		7.4
Medium	1,649.68		6.6
High	6,339.32		5.1

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 human Leptin assay, and the measured human 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
human Leptin	100%
mouse Leptin	0.3%
rat Leptin	≤0.1%
human Endothelin-1	<0.1%
human VEGF	<0.1%
human GROα	<0.1%
human SCF	<0.1%
human TPO	<0.1%

Sample Recoveries

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

Human Leptin concentrations were measured in a variety of different samples including tissue culture media, human serum and human EDTA plasma. Human Leptin was spiked into the undiluted samples of these media 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	98.9	≥1:2
Human EDTA plasma	105.0	≥1:8
Human serum	85.1	≥1:16

* See Sample Handling instructions on page 4 for details.

References

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

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