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User's Manual

Mouse/Rat Leptin ELISA Kit

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

This enzyme immunoassay kit is suited for measuring Leptin in mouse and rat serum for scientific purposes.

General Description

Leptin, the product of the ob gene, is a recently discovered single-chain proteohormone with a molecular weight of 16 kD which is thought to play a key role in the regulation of body weight. Its amino acid sequence exhibits no major homologies with other proteins. Leptin is almost exclusively produced by differentiated adipocytes. It acts on the central nervous system, in particular the hypothalamus, thereby suppressing food intake and stimulating energy expenditure. Leptin receptors - alternatively spliced forms exist that differ in length - belong to the cytokine class I receptor family. They are found ubiquitously in the body indicating a general role of leptin which is currently not fully understood. A circulating form of the leptin receptor exists which acts as one of several leptin binding proteins.

Besides its metabolic effects, leptin was shown to have a strong influence on a number of endocrine axes. In male mice, it blunted the starvation-induced marked decline of LH, testosterone, thyroxine and the increase of ACTH and corticosterone. In female mice, leptin prevented the starvation-induced delay in ovulation. Ob/ob mice, which are leptin deficient due to an ob gene mutation, are infertile. This defect could be corrected by administration of leptin, but not through weight loss due to fasting, suggesting that leptin is pivotal for reproductive functions.

All these actions may, at least in part, be explained by the suppressive effect of leptin on neuropeptide Y (NPY) expression and secretion by neurons in the arcuate nucleus. NPY is a strong stimulator of appetite and is known to be involved in the regulation of various pituitary hormones, e.g. suppression of GH through stimulation of somatostatin, suppression of gonadotropins or stimulation of the pituitary-adrenal axis.

The most important variable that determines circulating leptin levels is body fat mass. Obviously, under conditions of regular eating cycles, leptin reflects the proportion of adipose tissue showing an exponential relationship. This constitutive synthesis of leptin is modulated by a number of non-hormonal and hormonal variables. Stimulators in both rodents and humans are overfeeding, high fat diets, insulin and glucocorticoids. Suppression has been shown for fasting, cAMP and beta--3-adrenoceptor agonists. From these findings it becomes clear that leptin is an integral component of various metabolic and endocrine feedback loops.

Principles of Testing

The Mouse/Rat Leptin ELISA Kit is a so-called sandwich-assay. It utilizes two different specific high affinity polyclonal antibodies for this protein. The Leptin in the samples binds quantitatively to the immobilized antibody. In the following step, the biotinylated antibody in turn binds Leptin. After washing, a streptavidin-peroxidase-enzyme conjugate will be added, which will bind highly specific to the biotin of the antibody. Subsequently, the peroxidase catalyzes an enzymatic reaction resulting in a blue coloration. The intensity of the blue color depends on the Leptin content of the sample. The reaction is stopped by the addition of stop solution and color intensity is quantified by measuring the absorption.

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with goat anti-mouse/rat Leptin-antibody. Wells are separately breakable. (8x12) wells
2. **Standards (A-G):** lyophilized, (recombinant mouse Leptin), concentrations are given on vial labels and on quality certificate. 7 x 1 mL
3. **Control Serum (KS):** lyophilized, (mouse serum), concentration is given on quality certificate. 1 x 200 µL
4. **Antibody Conjugate (AK):** 100-fold concentrated, contains goat biotinylated anti-mouse/rat leptin antibody. 1 x 120 µL
5. **Enzyme Conjugate (EK):** 100-fold concentrated, contains HRP (Horseradish-Peroxidase)-labelled. 1 x 120 µL
6. **Dilution Buffer (VP):** ready for use. Please shake before use! 1 x 120 mL
7. **Washing Buffer (WP):** 20-fold concentrated solution. 1 x 50 mL
8. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised Tetramethylbencidine. 1 x 12 mL
9. **Stopping Solution (SL):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
10. **Sealing Tape:** for covering the microtiter plate. 3

Materials Required But Not Supplied

1. Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer WP (A. dest.), 950 mL.
2. Precision pipettes and multichannel pipettes with disposable plastic tips
3. Polyethylene PE/Polypropylene PP tubes for dilution of samples
4. Vortex-mixer
5. Microtiter plate shaker (350 rpm)
6. Microtiter plate washer (recommended)
7. Microplate reader ("ELISA-Reader") with filter for 450 and ≥590 nm

Storage

Store the kit at 2-8°C after receipt until its expiry date. The lyophilized reagents should be stored at –20 °C after reconstitution. Avoid repeated thawing and freezing.

The shelf life of the components after initial opening is warranted for 4 weeks, store the unused strips and microtiter wells airtight together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. Reconstituted components must be stored at –20°C (or colder). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Freezing is only possible once! The 1:20 diluted Washing Buffer WP is 4 weeks stable at 2-8°C.

Specimen Collection And Preparation

1. Sample type: Mouse and Rat Serum, Plasma.

Serum as well as Heparin-, EDTA- or Citrate-Plasma plasma are suitable samples. Possible dilution of the sample by the anticoagulant must be considered.

2. Specimen collection

Haemolytic reactions have to be avoided.

3. Required sample volume: According to Leptin level of the sample, maximal 50 µL per test. Samples should be diluted prior to measurement with Dilution Buffer VP depending on the expected values.

4. Sample stability

Undiluted serum specimen may be stored frozen at -20°C without loss of mouse/rat leptin. Repeated thawing and freezing should be avoided, although levels were found to be unaffected by a few cycles. Diluted samples are stable maximum of 2 h.

5. Sample dilution

Generally, a sample dilution of 1:5 to 1:20 is suitable. Depending upon species, stem and breeding and/or the individual experimental conditions this can, however, vary. If very low leptin concentrations are expected, 1:2 diluted samples might be used instead. However, if sample volume is limited, higher dilutions might be useful (provided that leptin concentration is sufficient).

• 1:5 with Dilution Buffer VP. E.g. for one double determination, pipette 200 µL Dilution Buffer VP in PE-/PP-Tube (application of a multi-stepper is recommended in larger series); add 50 µL sample (dilution 1:5). After mixing use 2 x 100 µL of this dilution in the assay.

• Or, pipette 80 µL Dilution Buffer VP in a well and add 20 µL Serum (mix well). Depending on the expected Leptin values the samples can be diluted higher in Dilution Buffer VP.

Reagent Preparation

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

1. The Standards A – G is reconstituted with 1 mL Dilution Buffer VP. After resuspension, the standards are diluted according to a gradient - A (25 pg/mL), B (50 pg/mL), C (100 pg/mL), D (200 pg/mL), E (400 pg/mL), F (800 pg/mL), G (1600 pg/mL), which are prepared for immediate use.
2. The Control KS is reconstituted with 200 µL Dilution Buffer VP. After reconstitution dilute the Control KS with the Dilution Buffer VP in the same ratio (1:5) as the sample.

Note: It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer. Thaw quickly but gently for further use (no temperature increases above room temperature and no excessive vortexing). Freezing is only possible once!

3. The required volume of Antibody Conjugate AK is prepared by 1:100 dilution of the provided 100-fold concentrate with Dilution Buffer VP.
4. The required volume of Enzyme Conjugate EK is prepared by 1:100 dilution of the provided 100-fold concentrate with Dilution Buffer VP.
5. The required volume of Washing Buffer WP is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest. The wash buffer WP, diluted 1:20, is stable for 4 weeks.

Assay Procedure

Note

1. Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate S, stabilised H_2O_2 Tetramethylbencidine, is photosensitive–store and incubation in the dark.
2. When performing the assay, Blank, Standards A-G, Control Serum KS and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody Conjugate AK, Enzyme Conjugate EK as well as the succeeding Substrate S should be added to the plate in the same order and in the same time interval as the samples. Stop Solution SL should be added to the plate in the same order as Substrate S. All determinations (Blank, Standards A-G, Control Serum KS and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
3. Shaking: The incubation steps should be performed at mean rotation frequency of a microtiter plate shaker. We recommend 350 rpm. Depending on the design of the shaker, the shaking frequency should be adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/ or false values, excessive shaking may result in high optical densities and/ or false values.
4. Washing: Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems. All washing must be performed with the provided Washing Buffer WP diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least. The danger of handling with potentially infectious material must be taken into account.

   Manual washing: Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. Decant contents into a biohazard bin, then blot plate on absorbent tissue. Wash the plate by adding 300µL Washing Buffer WP/well, then decant and blot on absorbent tissue. Repeat this step 2 more times for total of 3 washes.

Assay Step

1. Set Standards A-G, test samples (1:5 diluted), Control Serum KS (1:5 diluted) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate.
2. Aliquot 100ul of Dilution Buffer VP (Blank), Standards A-G, Control Serum KS and test samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 20-25°C, 350 rpm for 1 hour.
4. Wash: Aspirate the contents of the wells, and wash plate 3 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
5. Add 100ul Antibody Conjugate AK (1:100 diluted) into above wells. Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 20-25°C, 350 rpm for 1 hour.
6. Wash: Aspirate the contents of the wells, and wash plate 3 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
7. Add 100ul Enzyme Conjugate EK (1:100 diluted) into above wells. Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 20-25°C, 350 rpm for 30 minutes.
8. Wash: Aspirate the contents of the wells, and wash plate 3 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
9. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark for 30

minutes.

10. Stop: Add 100ul Stopping Solution SL into each well.
11. Measure the absorbance within 15 min at 450 nm, with ≥ 590 nm as reference wavelength.

Quality Control

The absorbance values of the blank should be below 0.25, these of standard G (1600 pg/ml) should be above 1.0.

The determined and calculated concentration of Control KS should be within the range of the concentration given on vial label.

Calculation

1. Calculate the mean absorbance value for the blank from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance of the blank from the mean absorbances of all other values.
3. Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
4. Recommendation: Calculation of the standard curve should be done by using a computer program because the curve is in general (without respective transformation) not ideally described by linear regression. A higher-grade polynomial, or four parametric logistic (4-PL) curve fit or non-linear regression are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).
5. The Leptin concentration in pg/ml of the samples can be calculated by multiplication with the respective dilution factor.

Exemplary calculation of Leptin concentrations

Sample dilution: 1:5

Measured extinction of your sample 0.794

Measured extinction of the blank 0.134

Your measurement program will calculate the Leptin concentration of the diluted sample automatically by using the difference of sample and blank for the calculation. You only have to determine the most suitable curve fit.

In this exemplary case the following equation is solved by the program to calculate the Leptin concentration in the sample:

$y = 0.00117x$

$x = 548.2$ pg/mL

If the dilution factor (1:5) is taken into account the Leptin concentration of the undiluted sample is

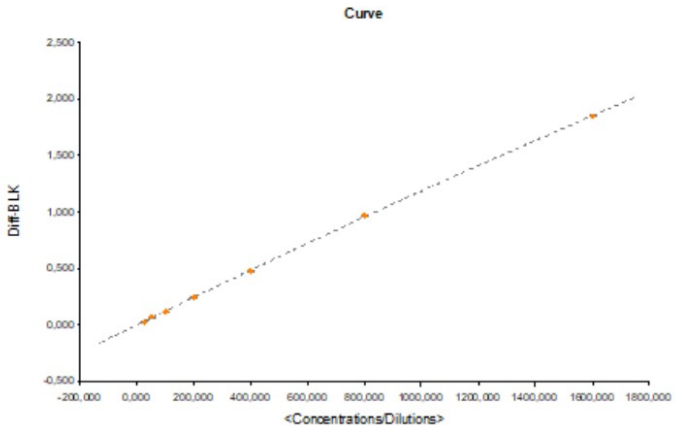
$548.2 \times 5 = 2741$ pg/mL

Typical Standard Curve

The following data is for demonstration only and cannot be used in place of data generation at the time of assay.

	A	B	C	D	E	F	G
ng/mL	25	50	100	200	400	800	1600
$OD_{(450\text{-}620\ nm)}$	0.031	0.072	0.116	0.244	0.483	0.968	1.841

The exemplary shown standard curve in Figure below cannot be used for calculation of your test results. You have to establish a standard curve for each test you conduct!



Precision

Inter-assay and intra-assay variation coefficients were found to be < 10 %.

Sensitivity

The practical sensitivity of the assay is 10 pg/ml, i.e., 1 pg/well (calculated by extrapolation of the standard curve).

Specificity

The Mouse/Rat Leptin ELISA Kit utilizes two specific high affinity polyclonal antibodies for these proteins. It recognizes quantitatively mouse leptin. Standards are prepared of recombinant mouse leptin. A certain degree of cross reactivity against rat leptin allows to use the kit also for measuring rat leptin. Dilutions of rat samples were found as linear as mouse samples. Preparations of recombinant mouse and rat leptin from the same producer were compared regarding their quantification with this kit. The relative potency of the rat material was found to be approx. 25%, compared to the respective mouse material, and, based on the nominal declaration of the producer. When working with rat samples, individual own calibrating of the kit values is recommended. The cross reactivity against human leptin is 0.7%.

Linearity

Dilution was found to be linear over the standard range.

Dilution	Serum 1 (pg/ml)	Serum 2 (pg/ml)	Serum 3 (pg/ml)	WHO NIBSC Code 97/626 (pg/ml)		
				nominal	1. Dilution results	2. Dilution results
1:2	1002	628	927	1500	1417.5	1609.3
1:4	1170	631	892	750	722.5	838.0
1:8	1212	601	855	375	343.6	397.3
1:16	1307	ND	ND	187.5	171.0	197.6
1:32	1424	ND	ND	93.75	88.4	98.2
1:64	1432	ND	ND	46.88	42.9	53.3
				23.44	21.0	ND

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Creative Diagnostics will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.
3. Do not use obviously damaged or microbial contaminated or spilled material.
4. This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.
5. Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.