Version 07-11/24

User's Manual

Human Leptin ELISA Kit

REF **DEIA-NB24-17**

Σ **96T**

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

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

The Leptin ELISA Kit is intended to be used for quantitative measurement of human Leptin in human serum and plasma samples.

In addition to serum and plasma samples Leptin can be determined in other human body fluids and in cell culture supernatants of human cell lines for research purposes.

General Description

Leptin, the product of the ob gene, is a 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. 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 by 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, 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.

For clinical purposes, it is important to note that serum leptin levels show a moderate circadian variation with a peak during the night at about 2 a.m.. The leptin values at this time are about 30 to 100% higher than the levels measured in the morning or early afternoon. This variation together with the influence of food intake needs to be taken into account, when blood samples are collected. Under fairly standardized conditions, i.e. normal eating cycles and blood sampling in the morning or early afternoon, a single leptin measurement is informative.

For the appropriate interpretation of measured leptin levels, reference ranges are required. Because body fat mass is the major confounding variable, these ranges should be referred to measures of the percentage body fat such as body mass index (BMI) or percent body fat determined by, e.g., bioelectric impedance assessment (BIA). Leptin levels are higher in females than in males and an age dependence was shown in children and adolescents. Therefore, reference ranges referring to measures of body fat should be stratified according to gender and pubertal development.

Leptin levels are high in most obese patients suggesting the presence of leptin insensitivity. In a small percentage of patients, however, leptin levels have been found inappropriately low with respect to their fat mass. It remains for future studies to prove that these patients represent a new pathophysiologic entity: leptin deficiency. Since leptin has also been shown to be of great importance for reproductive functions, possible new pathophysiologic mechanisms may be discovered relating infertility to insufficient leptin production.

The discovery of leptin has released an avalanche of research activities seeking to understand the regulation and actions of this hormone. Most importantly, it has provided a key to better understand the physiology of body weight regulation and to unveil possible pathophysiologic mechanisms in both obesity and eating disorders. Further, it may provide new insights into certain causes of infertility.

This enzyme immunoassay kit is suited for measuring human leptin in serum or plasma, and conditioned adipocyte culture media for scientific and diagnostic purposes. Measuring leptin in anorectic or cachectic patients, young children or in specimen other than serum, such as urine, cerebrospinal fluid, and certain cell culture media, is also possible with this kit.

The comparison with BMI-related reference ranges may be useful to detect conditions of relative leptin deficiency as a possible cause of obesity or provide an indication for leptin resistance respectively.

Principles of Testing

The Leptin ELISA Kit is a so-called Sandwich-Assay using two specific and highly affine antibodies. The Leptin in the samples binds to the first antibody coated on the microtiter plate. In the following step the second specific anti-Leptin-Antibody binds in turn to the immobilised Leptin. The second antibody is biotinylated and will be applied in a mixture with a Streptavidin-Peroxidase-Enzyme Conjugate. In the subsequent substrate reaction, the turn of the colour will be catalysed quantitatively depending on the Leptin level of the samples.

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with mouse-anti-Leptin-antibody. Wells are separately breakable. (8x12) wells
2. **Calibrators (CAL A-E):** lyophilized, (recombinant human Leptin), concentrations are given on vial labels and on quality certificate. 5 x 750 µL
3. **Control 1 (CTR1):** lyophilized, (human serum), concentration is given on quality certificate in ng/mL. 1 x 500 µL
4. **Control 2 (CTR2):** lyophilized, (human serum), concentration is given on quality certificate in ng/mL. 1 x 500 µL
5. **Antibody-HRP-Conjugate (DET):** ready for use, contains mouse-anti-hLeptin-antibody biotinylated + streptavidin horseradish peroxidase conjugate. 1 x 12 mL
6. **Dilution Buffer (DIL):** ready for use. Please shake before use! 1 x 25 mL
7. **Washing Buffer (WB):** 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 (STP):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
10. **Sealing Tape:** for covering the microtiter plate. 2

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. The reconstituted components calibrators A-E and Controls CTR1 and CTR2 must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Up to 3 of the freeze-thaw cycles did not influence the assay. The 1:20 diluted Washing Buffer WP is 4 weeks stable at 2-8°C

Specimen Collection And Preparation

1. Sample type: Serum and plasma

Beside serum also EDTA- and Heparin- plasma samples can be used because in five corresponding serum/plasma samples no difference between leptin concentrations of >30% was detected. Citrate plasma was also tested in two samples. The measured leptin concentration was 93 and 81% of the serum concentration. Thus, none of the tested anticoagulants interfered with the Leptin measurement.

2. Specimen collection

The blood sample for serum preparation should be gained according to standardized venipucture procedure. Hemolytic reactions have to be avoided.

Leptin levels show a circadian variation with a peak during the night at about 2 a.m.. This variation together with the influence of food intake needs to be taken into account, when blood samples are collected.

3. Required sample volume: 20 µL for single / 40 µL for duplicate measurement

4. Sample stability

In firmly closable sample vials

• Storage at 20-25°C: 2 days

• Storage at -20° C: min. 2 years

• Freeze-thaw cycles max. 5

The storage of samples over a period of 2 years at -20°C, showed no influence on the reading. Freezing and

thawing of samples should be minimized.

5. Interference

Hemoglobin, triglyceride and bilirubin in the sample do not interfere to a concentration of 1 mg/mL, 100 mg/mL and 100 µg/mL. However, the use of hemolytic, lipemic or icteric samples should be validated by the user.

6. Sample dilution

There is no special sample treatment necessary. The samples can be used undiluted (20 µL).

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 Calibrators A – E is reconstituted with 750 µL Dilution Buffer DIL. After resuspension, the calibrators are diluted according to a gradient - A (1 ng/mL), B (10 ng/mL), C (25 ng/mL), D (50 ng/mL), E (100 ng/mL), which are prepared for immediate use.
2. The Controls CTR1 and CTR2 are reconstituted with 500 µL Dilution Buffer DIL.

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.

3. The required volume of Washing Buffer WP is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest.

Assay Procedure

Note

1. Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate S, stabilised Tetramethylbencidine, is photosensitive–store and incubation in the dark.
2. When performing the assay, Blank, Calibrators A-E, Controls CTR1 and CTR2 and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody-HRP-Conjugate DET 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 STP should be added to the plate in the same order as Substrate S. All determinations (Blank, Calibrators A-E, Controls CTR1 and CTR2 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. 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 WB 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.

Automatic washing: When using an Automatic microtiter plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Manual washing: Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Assay Step

1. Set Calibrator A-E, test samples (undiluted), Controls CTR1 and CTR2 wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each calibrator and sample in duplicate.
2. Add 20ul Dilution Buffer DIL in all wells used. Aliquot 20ul of Dilution Buffer DIL (Blank), Calibrator A-E, Controls CTR1 and CTR2 and test samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 20-25°C, 200-350 rpm for 1h.
4. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WB. Do not let the wells dry completely at any time.
5. Add 100ul Antibody-HRP-Conjugate DET 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, 200-350 rpm for 30 minutes.
6. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WB. Do not let the wells dry completely at any time.
7. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark for 30 minutes.
8. Stop: Add 100ul Stopping Solution STP into each well.
9. Measure the absorbance within 30 min at 450 nm, with ≥ 590 nm as reference wavelength.

Quality Control

Good laboratory practice requires that controls are included in each assay. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The test results are only valid if the test has been performed following the instructions. Moreover, the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards/laws. All calibrators and kit controls must be found within the acceptable ranges as stated on the QC Certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

Quality criteria

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.25, and

the absorbance of Calibrator E should be above 1.00. Samples, which yield higher absorbance values than Calibrator E, should be re-tested with a dilution.

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 samples, controls and calibrators.
3. Plot the calibrator concentrations on the x-axis versus the mean value of the absorbance of the calibrators on the y-axis.
4. Recommendation: Calculation of the calibration 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 ng/mL of the samples and controls CTR1 and CTR2 is calculated automatically by your program.

Exemplary calculation of leptin concentrations

Measured extinction of your sample 0.39

Measured extinction of the blank 0.04

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(here: polynomial 3rd degree).

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

$0.35 = 5\times10^{-7}x^3-0.0002x^2 + 0.0346x – 0.0166$

$x = 7.012$

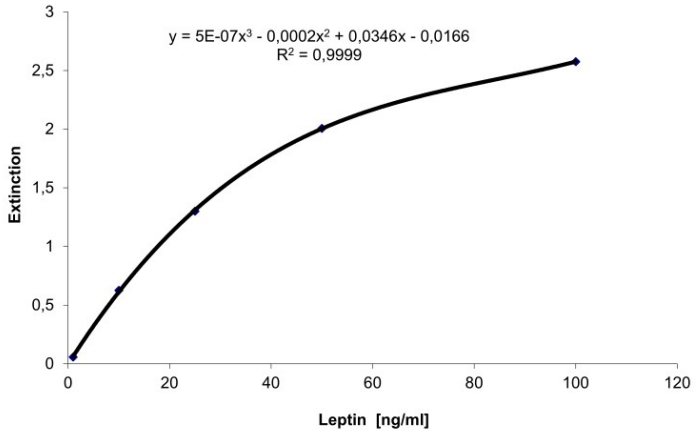
As the sample is undiluted the Leptin concentration of the sample is 7.012 ng/mL

Typical Standard Curve

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

	Blank	A	B	C	D	E
ng/mL	0	1	10	25	50	100
$OD_{(450\text{-}620\ nm)}$	0.04	0.083	0.68	1.449	2.165	2.764

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



Precision

Intra-Assay Variance

Two samples were measured eight times in three different assays. The measured coefficient of variation (CV) is 4.26% on average (n=6).

	Target value [ng/mL]	Determinations [n]	Mean value [ng/mL]	SD	CV [%]
Sample 1	85	8	90.36	4.68	5.18
Sample 2	6	8	6.42	0.3	4.71

Inter-Assay-Variability

Precision was evaluated by measuring the leptin content of the same serum samples several times in independent assays. On average the coefficient of variation was 12% (SD 3.5) and the deviation of the target value was less than 20% in 90% of the tested samples.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
Mean [ng/mL]	11.11	5.64	37.06	2.38	6.18	7.21	4.65	4.35	2.45	36.08	5.93	25.12	17.54
SD	0.92	0.57	3.26	0.43	0.64	0.57	0.49	0.61	0.47	3.68	0.75	2.63	1.66
CV [%]	8.33	10.10	8.81	17.97	10.35	7.88	10.57	14.04	19.21	10.19	12.57	10.45	9.46
n	121	99	28	182	86	86	79	23	27	25	22	26	31

Sensitivity

Limit of analytical sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the blank + 2SD. The analytical sensitivity of the Leptin ELISA Kit is <0.25 ng/ml (different determinations with range of 0.012 up to 0.24 ng/mL; with a mean of 0.095

ng/mL). Based on the undiluted sample the limit of quantification is 1 ng/mL.

Specificity

No signal was detected in serum of the following species: Horse, Cow, Chicken, Rabbit, Dog, Guinea pig, Sheep, Mouse, Goat, Donkey, Rat, Cat, Pig.

Linearity

Samples are routinely used undiluted. Dilution of 1:2 up to 1:20 have been tested with two serum samples and the recalculated amount of measured Leptin was not significant different of the amount in the undiluted sample. This however seems to be dependent on the sample. Two other samples were diluted up to 1:80 and showed insufficient linearity, thus a dilution higher than 1:10 is not recommended.

Dilution	Sample 1 [µg/L]	Sample 2 [µg/L]	Dilution	Sample 3 [µg/L]	Sample 4 [µg/L]
undiluted	35.2	17.8	undiluted	36.8	10.5
1:2	35.0	17.3	1:2	41.3	12.7
1:5	38.6	18.0	1:4	41.4	14.1
1:10	35.5	17.7	1:10	39.9	15.7
1:20	40.6	15.9	1:30	54.5	18.6
-	-	-	1:60	57.1	26.1
-	-	-	1:80	55.6	28.5
Mean [µg/L]	36.96	17.4	-	46.67	18.01
SD [µg/L]	2.5	0.86	-	8.65	6.86
CV [%]	6.8	4.97	-	18.54	38.06

Recovery

Recombinant leptin in Dilution Buffer (DIL) was used to enrich human serum samples. The leptin content of the so enriched samples was measured and recovery in comparison to the theoretical Leptin amount was calculated. Results are shown in table below and demonstrate that the two exemplary samples do not contain substances interfering with leptin measurement.

		Recombinant Leptin		Recovery [%]	
	w/o Leptin	5 µg/L	10 µg/L	5 µg/L	10 µg/L
Buffer	-	5.79	11.78	116	118
Sample 1	5.74	10.72	15.36	100	98
Sample 2	4.38	9.67	14.63	103	102

The results demonstrate that the two tested samples did not contain substances interfering with Leptin measurement. Further, the traceability of the test system to the international standard WHO/NIBSC 97/594 was evaluated. Therefore the NIBSC standard was diluted to 10, 20 and 40 ng/mL and used as sample. Recovery based on the nominal Leptin content of the NIBSC Standards was 106, 113 and 102% , respectively.

Interferences

Interference of bilirubin, hemoglobin and triglycerides was tested by adding different amounts of these substances to human serum containing Leptin. For comparison the same amount of buffer without any substance was also added to the serum. Table below demonstrates that neither bilirubin nor triglycerides or haemoglobin exert any influence on the measurement of Leptin in human serum.

	Triglyceride (100 mg/mL)	Bilirubin (100 µg/mL)	Hemoglobin (1 mg/mL)
Sample 1	95	101	94
Sample 2	107	105	105
Sample 3	111	101	101

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.
6. Following components contain human serum: Controls CTR1 and CTR2

   Source human serum for the Controls provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.

Limitations

The Leptin ELISA Kit is based on monoclonal antibodies. Generally, this technique could be sensible to heterophilic antibodies or rheumatic factors in the sample. Their influence is reduced by assay design, but cannot be excluded completely.