Version 07-11/24

**User's Manual**

# Biologically Active Leptin ELISA Kit

REF **DEIA-NB24-18**

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.

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# PRODUCT INFORMATION

## Intended Use

The Biologically Active Leptin ELISA Kit is intended to be used for quantitative measurement of functional, receptor binding Leptin levels in serum or plasma.

## General Description

Leptin a hormone of 146 amino acids shows a molecular weight of 16 kDa (P41159). It consists of four anti-parallel α-helices. Leptin is primarily produced by adipocytes and thus provides a signal to the energy state of the organism. By binding to the leptin receptor (P48357) it influences the activity of the JAK -STAT pathway and thereby regulates energy metabolism, especially the food intake. Leptin levels show a circadian variation (+/- 30%) and are dependent on BMI, pubertal status, and gender. In addition to the expression itself, the bioavailability of leptin, is regulated via a binding protein, as with many hormones with type I cytokine receptors. The binding protein is the extracellular domain of the Leptin receptor formed by proteolytic cleavage of the receptor be metalloproteases (ADAM 10/17).

In healthy persons food intake is reduced by increasing concentrations of leptin and by falling SOB-R quantities. In various pathological situations, this regulatory circuit is however interrupted: e.g. the leptin levels in the circulation of obese persons are increased, but resulting in no satiety. This phenomenon, known as leptin resistance, could have its cause in a reduction in the number of leptin receptors or in influencing the intracellular signal transduction by other parameters.

Lately it was shown that a naturally occurring transversion in the leptin gen (c.298G → T) results in an amino acid exchange of asparagine to tyrosin in position 100 (pD100Y). An in vitro cell culture model revealed that this mutated leptin was still secreted but unable to bind to the receptor and therewith unable to exert intracellular signaling. In vivo this mutation results in disturbance of food intake regulation and in consequence in extreme obesity. Under treatment with recombinant human leptin food intake behavior normalized and concomitantly body weight was reduced significantly.

Since the mutant leptin is recognized by the classical immunological leptin test systems, either a genetic test (sequencing of the ob gene), or a functional assay (binding to the receptor) is necessary for the diagnosis.

The Mediagnost bioLEP assay allows the measurement of leptin in human serum by binding it to the soluble receptor (leptin binding protein). Based on the measurement of receptor binding leptin the Mediagnost bioLEP assay provides additional information on the biological reactivity of the circulating leptin molecules.

Mutations resulting in less or no binding leptin can be detected by a significant reduction of the measured bioactive Leptin. Especially in connection with measurement of total Leptin by classical immunoassays (e.g. DEIA-NB24-17) the receptor-binding characteristics of leptin can easily be detected and quantified. This might help to evaluate mutation rates of the leptin gene by an inexpensive, fast method and accelerate obesity research by improved patient stratification in clinical studies.

## Principles of Testing

Recombinant produced leptin receptor (SOB-R) is immobilized on a microtiter plate. The sample to be tested

is diluted in dilution buffer and incubated on the microtiter plate; the leptin bound by the immobilized receptor is detected by a specific, polyclonal, Biotin-conjugated antibody and a streptavidin-conjugate. The quantification is performed with recombinant leptin, which is used as a standard and is traceable to the WHO International Standard (97/594).

## Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with human Leptin receptor (rec.). Wells are separately breakable. (8x12) wells
2. **Standards (A-H):** lyophilized, (recombinant Leptin), concentrations are given on vial labels and on quality certificate. 8 x 1 mL
3. **Control Serum 1 (KS1):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 500 µL
4. **Control Serum 2 (KS2):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 500 µL
5. **Antibody Conjugate (AK):** ready for use, contains biotinylated rabbit anti-hLeptin antibody. 1 x 12 mL
6. **Enzyme Conjugate (EK):** contains Streptavidin-Peroxidase Conjugate. 1 x 12 mL
7. **Dilution Buffer (VP):** ready for use. Please shake before use! 1 x 60 mL
8. **Washing Buffer (WP):** 20-fold concentrated solution. 1 x 50 mL
9. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised $H_2O_2$ Tetramethylbencidine. 1 x 12 mL
10. **Stopping Solution (SL):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
11. **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. The reconstituted components standards A-H and Control Sera KS1 and KS2 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

Serum and Heparin/EDTA plasma yield comparable values.

**2. Specimen collection**

Use standard venipuncture for the blood sampling. Haemolytic reactions have to be avoided.

**3. Required sample volume:** 15 µL

**4. Sample stability**

In firmly closable sample vials

• Storage at Room Temperature, 20-25°C: max. 2 days

• Storage at 4°C: max. 3 days

• Storage at -20°C: 2 years

• Freezer /-thaw cycles: max. 3

Freeze-thaw cycles should be minimized. Up to 3 cycles showed no effect on the measured Leptin concentration.

**5. Interference**

Neither triglycerides, bilirubin nor hemoglobin exert any influence on the measurement of Leptin in human serum up to concentrations of 100 mg/mL, 100 µg/mL and 1 mg/mL respectively.

**6. Sample dilution**

• Dilution: 1:20 with Dilution Buffer VP

• Example: Add 15 µL Sample to 285 µL Dilution Buffer VP (20 dilution factor).

• After dilution (1:20) samples may be stored at -20 ° C.

• Minimum required sample dilution 1:10

## 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 – H is reconstituted with 1 mL Dilution Buffer VP. After resuspension, the standards are diluted according to a gradient - A (0.0 ng/mL), B (0.05 ng/mL), C (0.15 ng/mL), D (0.5 ng/mL), E (1.25 ng/mL), F (2.5 ng/mL), G (4 ng/mL), H (6 ng/mL), which are prepared for immediate use.
2. The Control KS1 and KS2 are reconstituted with 500 µL Dilution Buffer VP. After reconstitution dilute the Control KS with the Dilution Buffer VP in the same ratio (1:20) 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.

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 $H_2O_2$ Tetramethylbencidine, is photosensitive–store and incubation in the dark.
2. When performing the assay, Blank, Standards A-H, Control Serum KS1/2 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-H, Control Serum KS1/2 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.

   **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 Standards A-H, test samples (1:20 diluted), Control Serum KS1/2 (1:20 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 Standards A-H, Control Serum KS1/2 and test samples into wells.

3. Incubate: Seal the plate with a cover and incubate at 20-25°C, 350 rpm for 2 hours.
4. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
5. Add 100ul Antibody Conjugate AK 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 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
7. Add 100ul Enzyme Conjugate EK 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 5 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 30 min at 450 nm, with ≥ 590 nm as reference wavelength.

## Quality Control

GLP requires that controls be run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. All 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.

**Quality criteria**

For the evaluation of the assay it is required that the absorbance values of the Standard A should be below 0.25, and the absorbance of standard H should be above 1.00. Samples, which yield higher absorbance values than Standard H, should be re-tested with a higher dilution.

## Calculation

1. Calculate the mean absorbance value for the Standard A from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance of the Standard A from the mean absorbances of all other samples and standards.
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 ng/mL (or pg/mL, according the chosen unit for the standards) of the samples and controls can be calculated by multiplication with the respective dilution factor.

**Exemplary calculation of sample concentrations**

Sample dilution: 1:20

Measured extinction of your sample 0.362

Measured extinction of Standard A 0.018

Your measurement program will calculate the Leptin concentration of the diluted sample automatically by using the difference of sample and Standard A 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:

$0.344 = 0.118x^2 + 1.7122x$

$x = 0.61$

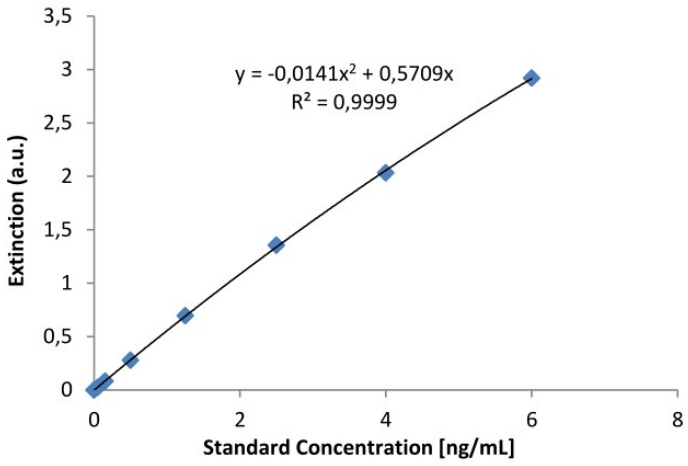
If the dilution factor (1:20) is taken into account the Leptin concentration of the undiluted sample is

$0.61 \times 20 = 12.21$ ng/mL

## Typical Standard Curve

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

| | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| ng/mL | 0.0 | 0.05 | 0.15 | 0.5 | 1.25 | 2.5 | 4 | 6 |
| $OD_{(450\text{-}620\ nm)}$ | 0.018 | 0.046 | 0.102 | 0.297 | 0.716 | 1.374 | 2.053 | 2.941 |




## Precision

### Intra-Assay Variance

Native human serum samples were measured repeatedly at various positions of the microtiter plate. Intra-Assay variability was on average < 5%.

| | Determinations [n] | Mean value [ng/mL] | SD | CV [%] |
|---|---|---|---|---|
| Sample 1 | 16 | 2.53 | 0.12 | 4.80 |
| Sample 2 | 16 | 33.05 | 0.79 | 2.39 |
| Sample 3 | 16 | 54.48 | 1.21 | 2.22 |

### Inter-Assay-Variability

Serum samples were measured in independent tests. The coefficient of variation was 9% on average.

| | Determinations [n] | Mean value [ng/mL] | SD | CV [%] |
|---|---|---|---|---|
| Sample 1 | 9 | 22.36 | 1.14 | 5.1 |
| Sample 2 | 9 | 2.54 | 0.21 | 8.4 |
| Sample 3 | 7 | 35.46 | 6.02 | 8.5 |

## Sensitivity

Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the blank + 2SD. The analytical sensitivity of the Biologically Active Leptin ELISA Kit is < 0.01 ng/mL. In 3 independent determinations values ranging from 0.0076 to 0.009 ng/mL were found.

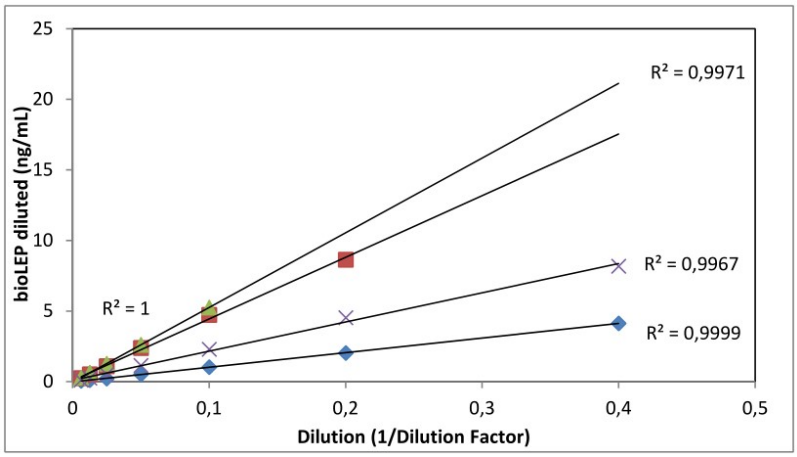
## Specificity

To determine the cross-reactivity with structurally similar proteins recombinant leptin, MYALEPT ™ (metreleptin) and recombinant leptin mutants D100Y and N103K, were used as samples. Recombinant leptin and Metreleptin were found to 100%, the maximum relative cross-reactivity with recombinant leptin mutants was < 0.5%.

| | total LEP [ng/mL] | bioLep [ng/mL] | % |
|---|---|---|---|
| Leptin-Mutation D100Y | 193.1 | 0.931 | 0.48 |
| Leptin-Mutation N103K | 37.1 | 0.031 | 0.09 |

## Linearity

The leptin concentration was determined at various dilutions of four human serum samples. The results shown in Figure below that in the samples tested no dilution effect can be demonstrated. The linearity is given from the dilution 1:10 to 1: 160.




## Recovery

Recovery of leptin international standard (NIBSC 97/594) or metreleptin was 102 and 109%, respectively. Recovery of spiked leptin or metreleptin in serum was 97 and 102 %, and leptin recovery by dilution experiments of serum was between 82 and 105 %.

The traceability of the Biologically Active Leptin ELISA Kit to an International Standard WHO/NIBSC 97/594 was examined. For this purpose the standard material was diluted to 0.6; 2.0; 6.0; and 8.8 ng/ml and used as a sample in the test. The recovery in relation to the nominal content of the NIBSC standards was on averaged 96.1%.

## Interferences

### Triclycerdies, Bilirubin and Hemoglobin

The interference physiologically occurring substances was tested by adding different amounts of these potentially interfering substances to serum samples. Table 6 shows the relative recovery of leptin in comparison to the serum without additions of these substances. None of the examined substances influenced the result of the test significantly.

| | Triglyceride (100 mg/mL) | Bilirubin (100 µg/mL) | Hemoglobin (1 mg/mL) |
|---|---|---|---|
| Sample 1 | 93 | 108 | 100 |
| Sample 2 | 99 | 107 | 96 |
| Sample 3 | 83 | 99 | 104 |

### Soluble Leptin Receptor (sOB-R)

Recombinant leptin was added in concentrations of 10 and 50 ng/mL to Dilutionpuffer VP and soluble leptin receptor (sOB-R) was added in concentrations of 20 - 100 ng/mL. The leptin contents were measured: Light sOB-R influence over mean leptin concentration was detected. This is negligible at high leptin concentration. Up to 100 ng/mL of soluble leptin receptor sOB-R does not interfere with the measurement of recombinant leptin.

| 10 ng /mL Leptin | | | 50 ng /mL Leptin | | |
|---|---|---|---|---|---|
| sOB-R | Recovery | | sOB-R | Recovery | |
| [ng/mL] | [ng/mL] | [%] | [ng/mL] | [ng/mL] | [%] |
| 0 | 10.5 | 104.6 | 0 | 50.5 | 100.9 |
| 20 | 9.2 | 94.8 | 20 | 50.8 | 101.6 |
| 40 | 9.2 | 91.5 | 40 | 53.1 | 106.2 |
| 60 | 9.0 | 90.4 | 60 | 51.2 | 102.5 |
| 80 | 8.8 | 87.7 | 80 | 51.6 | 103.2 |
| 100 | 8.7 | 87.4 | 100 | 50.3 | 100.6 |

## 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: Control Serum KS1 and KS2

   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.