Leptin (Mouse/Rat) ELISA

For the quantitative determination of leptin in mouse or rat serum and plasma.

*Please read carefully due to Critical Changes, e.g., plate shaker is required.*

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 22-LEPMS-E01  
Size: 96 wells  
TECHNICAL FEATURES

- Highly specific, sensitive and fast assay for mouse and rat leptin
- Calibrated against the WHO International Standard for mouse leptin: Code 97/626 (39, 40)
- No extraction required - No interference from leptin binding proteins due to high affinity of antiserum
- Complete recovery, measures total leptin in serum and plasma
- High sensitivity allows precise measurement also in lean animals: Detection limit 10 pg/ml
- Small sample volume requirement due to high sensitivity: e.g., less than 5 µl/well

INTRODUCTION

Leptin, the product of the ob gene (1,2), 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 (1). Leptin is almost exclusively produced by differentiated adipocytes (3-5). It acts on the central nervous system, in particular the hypothalamus, thereby suppressing food intake and stimulating energy expenditure (2,6-9). Leptin receptors - alternatively spliced forms exist that differ in length - belong to the cytokine class I receptor family (10-12). They are found ubiquitously in the body (10,11,13,14) 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 (15).

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 (16). 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 (17), 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 (6,18,19). NPY is a strong stimulator of appetite (20,21) and is known to be involved in the regulation of various pituitary hormones, e.g. suppression of GH through stimulation of somatostatin (22,23), suppression of gonadotropins (23) or stimulation of the pituitary-adrenal axis (21).

The most important variable that determines circulating leptin levels is body fat mass (24-26). Obviously, under conditions of regular eating cycles, leptin reflects the proportion of adipose tissue (27) showing an exponential relationship (37). This constitutive synthesis of leptin is modulated by a number of non-hormonal and hormonal variables. Stimulators in both rodents and humans are overfeeding (28,29), high fat diets (41), insulin (3,5,30-33) and glucocorticoids (5,34-36). Suppression has been shown for fasting (27), cAMP and beta--3-adrenoceptor agonists (35). From these findings it becomes clear that leptin is an integral component of various metabolic and endocrine feedback loops (38).
PRECAUTIONS AND WARNINGS
This kit is only for research use only, not for internal application of humans or animals. This product has to be used as described in the enclosed package insert. ALPCO is not liable or responsible for any damage or loss caused by non-observance of product instructions.

Following components **AK, EK, VP, A-E** contain **0.01% 2-Methyl-4-isothiazolin-3-one Solution** as preservative
- **R34** Irritating to eyes and skin
- **R43** Sensibilisation through skin contact possible
- **S26** In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
- **S36/37** Wear suitable protective clothing and gloves
- **S45** In case of accident or if you feel unwell seek medical advice

Following components **AK, EK, VP, WP, A-E** contain **0.01% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one und 2-methyl-2H-isothiazol-3-one as preservative**
- **R36/38** Irritating to eyes and skin
- **R43** Sensibilisation through skin contact possible
- **S26** In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
- **S28.1** After contact with skin, wash immediately with plenty of water

**Substrat (S)**
TMB-Substrat (S) contains **3,3’,5,5´ Tetramethylbenzidine.**
- **R20/21/R22** Harmful by inhalation, in contact with skin and if swallowed
- **R36/37/38** Irritating to eyes, respiratory system and skin
- **S26** In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
- **S36/37** Wear suitable protective clothing and gloves

**Stop Solution (SL)**
Stop solution contains **0,2 M Sulfur Acid (H₂SO₄)**
- **R36/38** Irritating to eyes and skin
- **S26** In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
- **S36/37** Wear suitable protective clothing and gloves.

The kit should not be used beyond the expiration date on the kit label.
All reagents are for research use only!
In conducting the assay, follow strictly the test protocol.
The acquisition, possession and use of the kit are subject to the regulations of the national regulatory authorities.
Reagents with different lot numbers should not be mixed.
First aid procedures:

*Skin contact:* Wash affected area thoroughly with water. Discard contaminated cloths and shoes.

*Eye contact:* In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing spread the eyelids.

*Ingestion:* If swallowed, wash out mouth thoroughly with water. Immediately see a physician. The Stop Solution provided is an acid solution. Avoid direct contact. Wear eye, hand, face and clothing protection when using this material.

The handling of potentially infectious material must comply with the following guidelines:

Do not eat, drink or smoke in these areas.

Never pipette the materials with the mouth.

Spilled material must be wiped off immediately and should become disinfected. Clean contaminated areas and equipment with a suitable detergent.

**TECHNICAL RECOMMENDATIONS**

Lyophilized components of the kit should be stored at 2 – 8°C. They are stable as indicated on the respective labels.

The shelf life of the components after opening is not affected, if used appropriately.

Bring all reagents to room temperature (20 - 25°C) before use.

For reconstitution of lyophilized components the kit, Dilution Buffer VP should be used. It is recommended to keep reconstituted reagents at room temperature for half an hour, and then to mix them thoroughly but gently (no foam should result), e.g., with a Vortex mixer.

After reconstitution, components should be stored at 4 –8 °C for up to 1 week. If longer storage time is needed, store the components frozen at –20°C or below. However, this can be done only once! Avoid repeated freeze-thaw cycles. In case you plan to perform multiple independent determinations over a longer period with one kit, you should aliquot the components prior to freezing into suitable smaller volumes. This is strongly recommended.

When performing the assay, the Standards S, Control KS and the samples should be pipetted as fast as possible (e.g., 15 minutes). Antibody Conjugate AK should be added to the plate in the same order and the same time interval as the samples. Stop Solution should be added to the plate in the same order as the Substrate Solution.

**Room temperature incubation means incubation at 20 – 25°C.**

The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must become 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.

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 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. When using an automatic microtiter plate washer, the respective instructions for 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 is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamically 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.

CALIBRATION OF THE ASSAY

The Mouse/Rat Leptin ELISA has been calibrated against the International Reference Standard for mouse leptin. The definition of this international reference material, code 97/626, was evaluated in an international collaborative study, with this test kit (39). The standard preparation of the WHO with code 97/626 (39) is available from the NIBSC (40).

One ampoule of the preparation, reconstituted in 1 ml solution, will be quantified with this kit to the nominal content of 4000 ng mouse leptin.

ASSAY CHARACTERISTICS AND VALIDATION

The ELISA for Mouse/Rat Leptin utilizes two specific high affinity polyclonal antibodies for these proteins. It recognizes quantitatively mouse leptin. Standards are prepared of recombinant mouse leptin.

A high degree of cross-reactivity against rat leptin allows the use of 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 plus 95%, 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. This kit is calibrated against the WHO NIBSC mouse leptin standard code 97/626 (see above).

The cross-reactivity against human leptin is 0.7%.

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

Inter-assay and intra-assay variation coefficients were found to be < 4.7% and < 4.4%, respectively. Sample dilution was found to be linear over the standard range. Exemplary determinations are shown in the tables 1, 2 and 3.
Table 1: Inter-Assay-Variation: Different sera, independently diluted, measured in duplicate

<table>
<thead>
<tr>
<th>Sample 1 (pg/ml)</th>
<th>867.9</th>
<th>802.8</th>
<th>874.1</th>
<th>822.5</th>
<th>904.5</th>
<th>821.8</th>
<th>901.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 2 (pg/ml)</td>
<td>1208.2</td>
<td>1169.6</td>
<td>1306.5</td>
<td>1275.6</td>
<td>1276.8</td>
<td>1212.0</td>
<td>1246.5</td>
</tr>
<tr>
<td>Sample 3 (pg/ml)</td>
<td>627.9</td>
<td>631.2</td>
<td>601.0</td>
<td>638.7</td>
<td>590.2</td>
<td>612.2</td>
<td>586.3</td>
</tr>
</tbody>
</table>

Table 2: Intra-Assay-Variation: Different sera independently 1:5 diluted, measured 6-fold each in duplicate

<table>
<thead>
<tr>
<th>Sample 1 (pg/ml)</th>
<th>1262</th>
<th>1266</th>
<th>1197</th>
<th>1260</th>
<th>1218</th>
<th>1247</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 2 (pg/ml)</td>
<td>869</td>
<td>829</td>
<td>790</td>
<td>826</td>
<td>790</td>
<td>821</td>
</tr>
<tr>
<td>Sample 3 (pg/ml)</td>
<td>436</td>
<td>457</td>
<td>464</td>
<td>454</td>
<td>423</td>
<td>418</td>
</tr>
</tbody>
</table>

Table 3: Linearity of the sample dilution: Independent assays, independent dilutions as indicated, determinations in duplicate

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Serum 1 (pg/ml)</th>
<th>Serum 2 (pg/ml)</th>
<th>Serum 3 (pg/ml)</th>
<th>WHO NIBSC Code 97/626 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nominal</td>
</tr>
<tr>
<td>1:2</td>
<td>1002</td>
<td>628</td>
<td>927</td>
<td>1500</td>
</tr>
<tr>
<td>1:4</td>
<td>1170</td>
<td>631</td>
<td>892</td>
<td>750</td>
</tr>
<tr>
<td>1:8</td>
<td>1212</td>
<td>601</td>
<td>855</td>
<td>375</td>
</tr>
<tr>
<td>1:16</td>
<td>1307</td>
<td>n.d.</td>
<td>n.d.</td>
<td>187.5</td>
</tr>
<tr>
<td>1:32</td>
<td>1424</td>
<td>n.d.</td>
<td>n.d.</td>
<td>93.75</td>
</tr>
<tr>
<td>1:64</td>
<td>1432</td>
<td>n.d.</td>
<td>n.d.</td>
<td>46.88</td>
</tr>
</tbody>
</table>

23.44   | 21.0            | n.d.            |

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MATERIALS

Materials Provided

1) **Microtiter plate**, ready for use: Microtiter plate with 96 wells, divided up in 12 strips with 8 wells separately breakable, coated with anti-mouse/rat leptin antibody and packed in a laminate bag.

2) **Standards A-G**, lyophilized: Standard concentrations between 25 and 1600 pg/ml recombinant mouse leptin. Reconstitute with **1 ml Dilution Buffer VP** each. Exact concentrations are given on the labels of the vials.

3) **Control KS**, lyophilized: Mouse serum, the exact mouse leptin concentration and the acceptable range is given on the vial label. Reconstitute with **200 µl Dilution Buffer VP**. Dilute according to the dilution of serum samples.

4) **Antibody Conjugate AK**, 120 µl, 100-fold concentrated: Biotinylated anti-mouse/rat leptin antiserum. Dilute **1:100** with **Dilution Buffer VP**.

5) **Enzyme Conjugate EK**, 120 µl, 100-fold concentrated: Horse-radish peroxidase conjugated streptavidin. **Dilate immediately before use 1:100 with Dilution Buffer VP.**

6) **Dilution Buffer VP**, 120 ml, ready for use.

7) **Washing buffer WP**, 50 ml, 20-fold concentrated: Dilute 1:20 with distilled water before use. Attention: After dilution the **Washing Buffer** is only 4 weeks stable, please dilute only according to requirements.

8) **TMB-substrate solution S**, 12 ml, ready for use.

9) **Stopping solution SL**, 0.4 N sulphuric acid, 12 ml, ready for use.

10) **Sealing tape** for covering of the microtiter plate, 2 x foils.

Materials not Provided

- Precision pipettes and multichannel pipettes with disposable plastic tips
- Distilled or deionized water for dilution of the Washing Buffer (WP)
- Vortex-mixer
- Microtiterplate shaker (350 rpm)
- Microtiterplate washer (recommended)
- Micro plate reader ("ELISA-Reader") with filter for 450 and ≥590 nm
- Polyethylen PE/Polypropylene PP tubes for dilution of samples

**Kit Components and Sample Preparation**

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

Ensure that lyophilized materials are completely dissolved on reconstitution (see Technical Recommendations).

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.

Samples should be diluted prior to measurement with Dilution Buffer VP depending on the expected values.

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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 (or even undiluted samples) might be used instead. However, if sample volume is limited, higher dilutions might be useful (provided that leptin concentration is sufficient).

Example for the 1:5 dilution
Please pipette 200 µl dilution Buffer VP in PE/PP-Tubes (application of a multi-stepper is recommended in larger series); subsequently add 50 µl serum or plasma samples (dilution 1:5). After mixing use 100 µl of this dilution per determination. Or, pipette 80 µl buffer in a well and add 20 µl Serum (mix well).

ASSAY PROCEDURE
For optimal results, accurate pipetting and adherence to the protocol are recommended. Due to usual general considerations in performing ELISAs, Standards and Samples should be assayed in duplicate. Proposed pipetting plan (example):

1) Add 100 µl dilution buffer VP in wells A1/A2 (blank) and
2) pipette in position B1/B2 100 µl standard A,
pipette in position C1/C2 100 µl standard B,
pipette in position D1/D2 100 µl standard C,
pipette in position E1/E2 100 µl standard D,
pipette in position F1/F2 100 µl standard E,
pipette in position G1/G2 100 µl standard F,
pipette in position H1/H2 100 µl standard G.

100 µl each of the diluted Control KS should be pipetted into wells A3/A4.
100 µl each of the diluted Samples can be pipetted in all other wells, e.g. Sample 1 in well B3/B4, etc.

To avoid distortions due to differences in incubation times, standards, samples and controls should be pipetted as fast as possible.
3) Cover the wells with sealing tape and incubate the plate for 1 hour at room temperature (shake at 350 rpm).
4) After incubation aspirate the contents of the wells into a disinfectant (due to the possible theoretical risk of infection!) and wash the wells 3 times with 300 µl of Washing Buffer WP / well respectively. The Washing Buffer WP should incubate at least for 15 seconds/cycle
5) Pipette 100 µl of the diluted Antibody Conjugate AK solution in each well, cover the wells with sealing tape and incubate the plate for another 1 hour at room temperature (shake at 350 rpm).
6) After incubation wash the wells 3 times with Washing Buffer WP as described above.
7) Pipette 100 µl of the diluted Enzyme Conjugate EK solution in each well, cover the wells with sealing tape and incubate the plate for another 30 minutes at room temperature (shake at 350 rpm).
8) After incubation wash the wells 3 times with Washing Buffer WP as described above.
9) Pipette 100 µl of the TMB-substrate solution S in each well, incubate the plate for 30 minutes at room temperature in the dark.
10) Stop the reaction by adding 100 µl of Stopping Solution SL in each well.
11) Measure the absorbance within 15 minutes at 450 nm (reference filter: ≥ 590 nm).
EVALUATION OF RESULTS

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.

Establishing the Standard Curve

The standards provided contain the following concentrations of recombinant mouse leptin:

<table>
<thead>
<tr>
<th>Standard:</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>mLEPTIN (pg/ml):</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>800</td>
<td>1600</td>
</tr>
</tbody>
</table>

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.

REFERENCES


23. Pierroz DD, Catzelflis C, Aebl AC, Rivier JE, Aubert ML. 1996 Chronic administration of neuropeptide Y into the lateral ventricle inhibits both the pituitary-testicular axis and growth hormone and insulin-like growth factor I secretion in intact adult male rats. Endocrinol. 137:3-12. 


40. Address NIBSC: National Institute for Biological Standards and Controls, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

Summary of the Assay

<table>
<thead>
<tr>
<th>Reagent preparation:</th>
<th>Reconstitution:</th>
<th>Dilution:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards A-G</td>
<td>in 1 ml Dilution Buffer VP</td>
<td></td>
</tr>
<tr>
<td>Antibody Conjugate AK</td>
<td>1:100 with Dilution Buffer VP</td>
<td></td>
</tr>
<tr>
<td>Enzyme Conjugate EK</td>
<td>1:100 with Dilution Buffer VP</td>
<td></td>
</tr>
<tr>
<td>Control Serum KS</td>
<td>in 200 µl Dilution Buffer VP</td>
<td>e.g.: 1:5 with Dilution Buffer VP</td>
</tr>
<tr>
<td>Washing Buffer WP</td>
<td>1:20 with Aqua. dest. (e.g., add the complete contents of the flask (50 ml) into a graduated flask and fill with A.dest. to 1000 ml).</td>
<td></td>
</tr>
</tbody>
</table>

Sample dilution: e.g. 1:5 (Mix 50 µl Serum with 200 µl Dilution Buffer VP) or 1:10, 1:20 etc.
Before conducting the assay equilibrate all reagents to room temperature.

Assay Procedure for Double Determination

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Reagents</th>
<th>Well positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µl</td>
<td>Dilution Buffer VP</td>
<td>A1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Standard A (25 pg/ml)</td>
<td>B1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Standard B (50 pg/ml)</td>
<td>C1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Standard C (100 pg/ml)</td>
<td>D1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Standard D (200 pg/ml)</td>
<td>E1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Standard E (400 pg/ml)</td>
<td>F1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Standard F (800 pg/ml)</td>
<td>G1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Standard G (1600 pg/ml)</td>
<td>H1/2</td>
</tr>
<tr>
<td>100 µl</td>
<td>Control Serum KS</td>
<td>A3/4</td>
</tr>
<tr>
<td>100 µl</td>
<td>Samples (diluted)</td>
<td>following wells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cover the wells with the sealing tape.

**Incubation: 1 h at RT, 350 rpm**

| 3x 300 µl | Aspirate the contents of the wells and wash 3x with 300 µl Wash Buffer WP | each well |
| 100 µl    | 1:100 diluted Antibody Conjugate AK | each well |

**Incubation: 1 h at RT, 350 rpm**

| 3x 300 µl | Aspirate the contents of the wells and wash 3x with 300 µl Wash Buffer WP. | each well |
| 100 µl    | 1:100 diluted Enzyme Conjugate EK | each well |

**Incubation: 30 min at RT, 350 rpm**

| 3x 300 µl | Aspirate the contents of the wells and wash 3x with 300 µl Wash Buffer WP. | each well |
| 100 µl    | Substrate Solution S | each well |

**Incubation: 30 min in the dark at RT**

| 100 µl | Stop Solution SL | each well |

Measure the absorbance within 15 min at 450 nm with ≥590 nm as reference wavelength.