

m/rLeptin-ELISA Kit

**Enzyme-Immunoassay for Quantitative
Determination of**

Mouse and Rat Leptin (Obese Protein)

**Product Code: E06
(96 Determinations)**

**For In-Vitro Use Only!
For Research Use Only!**



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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), 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

Mediagnost E06 Kit is only for use in in vitro Diagnostics, **not** for internal application of humans or animals. This product has to be used as described in the enclosed package insert. Mediagnost GmbH 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 in vitro use only!

In conducting the assay, follow strictly the test protocol.

The acquisition, possession and use of the kit is 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.

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.

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

If no microtiter plate shaker is available, please refer to the alternative assay procedure given below.

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.

CALIBRATION OF THE ASSAY

The Mediagnost Mouse/Rat Leptin ELISA E06 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 Mediagnost test kits and with participation of Mediagnost (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 E06 to the nominal content of 4000 ng mouse leptin.

ASSAY CHARACTERISTICS AND VALIDATION

The ELISA for Mouse/Rat Leptin E06 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 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 E06. 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. E06 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

Sample 1 (pg/ml)	867.9	802.8	874.1	822.5	904.5	821.8	901.9
Sample 2 (pg/ml)	1208.2	1169.6	1306.5	1275.6	1276.8	1212.0	1246.5
Sample 3 (pg/ml)	627.9	631.2	601.0	638.7	590.2	612.2	586.3

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

Sample 1 (pg/ml)	1262	1266	1197	1260	1218	1247
Sample 2 (pg/ml)	869	829	790	826	790	821
Sample 3 (pg/ml)	436	457	464	454	423	418

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

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	n.d.	n.d.	187.5	171.0	197.6
1:32	1424	n.d.	n.d.	93.75	88.4	98.2
1:64	1432	n.d.	n.d.	46.88	42.9	53.3
				23.44	21.0	n.d.

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. **Dilute 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

- Distilled or demineralized water for dilution of the washing buffer WP
- Micropipettes and multichannel pipettes with disposable plastic tips
- Vortex-mixer (recommended)
- Device to aspirate the standards and the samples from the wells
- Microtiterplate washer and shaker (recommended)
- Microplate reader ("ELISA-Reader") with filter for 450/620nm wavelength
- Foil welding device for laminate bags (recommended)

ASSAY PROCEDURE

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.

Serum samples should be diluted prior to measurement with Dilution Buffer VP depending on the expected values. For **rat samples** a dilution of **1:5** or **1:10** is generally suitable. For **mouse samples** a dilution of **1:20 should be applicable**.

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).

Example for the 1:5 dilution

Proposal: Mix 50 µl serum with 200 µl dilution buffer **VP** (1: 5). Use 2 x 100 µl of this dilution in the assay.

Or, pipette 80 µl buffer in a well and add 20 µl Serum (mix well – only recommended if a special microtiter plate shaker is available).

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 **100µl standard F**,
pipette in position H1/H2 **100µl 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. Incubate at room temperature.

- 3) Cover the wells with sealing tape and incubate the plate for **1 hour**, shake with **350 rpm**.
- 4) After incubation aspirate the contents of the wells into a disinfectant (possible theoretically risk of infection!) and wash the wells **3 times** with **250 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**, shake with **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**, shake with **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** 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).

Alternative procedure

If no microtiter plate shaker is available, the test procedure should be adapted as follows. Incubation without shaking reduces the optical densities. This can be corrected by prolongation of the incubation steps 3) and 5), i.e., Standards **A-G** and samples as well as Antibody Conjugate **AK** should be incubated for 2 hours each at room temperature. This will correct the loss of optical densities due to incomplete binding reactions. All other steps should be performed as described above.

EVALUATION OF RESULTS

The absorbance values of the blank should be below 0.2, these of standard G (1600 pg/ml) should exceed 1.5.

The determined and calculated concentration of Control **KS** should be within the range of the concentration given on vial label ($\pm 2SD$).

Establishing the Standard Curve

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

Standard:	A	B	C	D	E	F	G
mLEPTIN (pg/ml):	25	50	100	200	400	800	1600

- 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**.

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Summary of the Assay

Reagent preparation:	Reconstitution:	Dilution:
Standards A-G	in 1 ml Dilution Buffer VP	
Antibody Conjugate AK		1:100 with Dilution Buffer VP
Enzyme Conjugate EK		1:100 with Dilution Buffer VP
Control Serum KS	in 200 µl Dilution Buffer VP	e.g. 1:5 with Dilution Buffer VP
Washing Buffer WP		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).
Sample dilution: e.g. 1:5 (Mix 50 µl Serum with 200 µl Dilution Buffer VP) or 1:10, 1:20 etc.		

Assay Procedure for Double Determination

Pipette	Reagents	Well positions
100 µl	Dilution Buffer VP	A1/2
100 µl	Standard A (25 pg/ml)	B1/2
100 µl	Standard B (50 pg/ml)	C1/2
100 µl	Standard C (100 pg/ml)	D1/2
100 µl	Standard D (200 pg/ml)	E1/2
100 µl	Standard E (400 pg/ml)	F1/2
100 µl	Standard F (800 pg/ml)	G1/2
100 µl	Standard G (1600 pg/ml)	H1/2
100 µl	Control Serum KS	A3/4
100 µl	Sample dilution	following wells
Cover the wells with the sealing tape.		
Incubation: 1 h at RT, ≥ 350 rpm		
3x 250 µl	Aspirate the contents of the wells and wash 3x with 250 µl Wash Buffer WP	each well
100 µl	1:100 diluted Antibody Conjugate AK	each well
Incubation: 1 h at RT, ≥350 rpm		
3x 250 µl	Aspirate the contents of the wells and wash 3x with 250 µl Wash Buffer WP .	each well
100 µl	1:100 diluted Enzyme Conjugate EK	each well
Incubation: 30 min at RT, ≥350 rpm		
3x 250 µl	Aspirate the contents of the wells and wash 3x with 250 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.		