m/rLeptin-ELISA Kit

Enzyme-Immunoassay for Quantitative Determination of

Mouse and Rat Leptin (Obese Protein)

Product Code: KAPME06
(96 Determinations)

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

KAPME06 Kit is only for use in vitro Diagnostics, **not** for internal application of humans or animals. This product has to be used as described in the enclosed package insert. DIAsource is not liable or responsible for any damage or loss, caused by non-observance of product instructions.

Kathon CG
contained in: **Biotin conjugate, HRP conjugate, Dilution buffer, Washing buffer, Standards**
< 0.1% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-isothiazol-3-one

ProClin® 950
contained in: Biotin conjugate, HRP conjugate, Dilution buffer, Washing buffer
<0.1% 2-Methyl-4-isothiazolin-3-one

Chromogenic Solution
TMB-Substrat 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

Stopping Solution
Stopping solution contains 0,2 M Sulfur Acid (H_2SO_4)

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 throughly with water. Immediately see a physician.The Stopping 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 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 between 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, Control 1 and the samples should be pipetted as fast as possible (e.g., 15 minutes). Biotin conjugate should be added to the plate in the same order and the same time interval as the samples. Stopping Solution should be added to the plate in the same order as the Chromogenic substrate.

CALIBRATION OF THE ASSAY

The Mouse/Rat Leptin ELISA KAPME06 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 (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 KAPME06 to the nominal content of 4000 ng mouse leptin.

ASSAY CHARACTERISTICS AND VALIDATION

The ELISA for Mouse/Rat Leptin KAPME06 utilizes two specific high affinity polyclonal antibodies for these proteins. It recognizes quantitatively mouse leptin. Standards are prepared with 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 KAPME06. 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. KAPME06 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 1-7,** lyophilized: Standard concentrations between 25 and 1600 pg/ml recombinant mouse leptin. Reconstitute with **1 ml Dilution Buffer** each. Exact concentrations are given on the labels of the vials**.**

3) **Control 1,** lyophilized: Mouse serum, the exact mouse leptin concentration and the **acceptable range** is given on the vial label. Reconstitute with **200 µl Dilution Buffer**. Dilute according to the dilution of serum samples.

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

5) **HRP conjugate**, 120 µl, 100-fold concentrated: Horseradish peroxidase labelled streptavidin. **Dilute immediately before use 1:100 with Dilution Buffer**.

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

7) **Washing buffer,** 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) **Chromogenic Substrate,** 12 ml, ready for use**.**

9) **Stopping solution,** 0.4 N sulfuric 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
- 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 at 350 rpm (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 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 depending on the expected values. **Usually a dilution of 1: 5 is appropriate**.

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) 1:20 or above may be used – i.e., 5 µl serum or less per well.

Suggestion for dilution protocol

Proposal: Mix 50 µl serum with 200 µl dilution buffer (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 **100µl** dilution buffer in wells A1/A2 (blank) and
2) pipette in position B1/B2 **100 µl standard 1**,
   pipette in position C1/C2 **100 µl standard 2**,
   pipette in position D1/D2 **100 µl standard 3**,
   pipette in position E1/E2 **100 µl standard 4**,
   pipette in position F1/F2 **100 µl standard 5**,
   pipette in position G1/G2 **100 µl standard 6**,
   pipette in position H1/H2 **100 µl standard 7**.

100 µl each of the diluted **Control 1** 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** / well respectively. The washing buffer should incubate at least for 15 seconds/cycle
5) Pipette **100 µl** of the **diluted Biotin conjugate** 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** as described above.
7) Pipette **100 µl** of the **diluted HRP conjugate** 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** as described above.
9) Pipette **100 µl** of the **chromogenic substrate** in each well, incubate the plate for **30 minutes** in the dark.
10) Stop the reaction by adding **100 µl** of **Stopping Solution** 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 **1-7** and samples as well as biotin conjugate 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 7 (1600 pg/ml) should exceed 1.5.

The determined and calculated concentration of Control 1 should be within the range of the concentration given on vial label (±2SD).

Standard Curve

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

Standard:	**1**	**2**	**3**	**4**	**5**	**6**	**7**
mLEPTIN (pg/ml):	25	50	100	200	400	800	1600

1) Calculate the mean absorbance value for each duplicate and substract the absorbance of the blank (well A1/A2).
2) Plot the standard concentrations 1-7 against absorbance values of each standard. Calculation of standard curve should be done by using a computer programme. The standard curve regression usually is best described by four **parameter logistic (4PL)** lin-log curve fit, or, use another appropriate regression. The measured absorbance values can then easily be used to calculate the mouse leptin concentration of the samples (by multiplication with the respective dilution factor).

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Summary of the Assay

Reagent preparation:	**Reconstitution:**	**Dilution:**
Standards 1-7	in **1 ml** Dilution Buffer	
Biotin conjugate		**1:100** with Dilution Buffer
HRP conjugate		**1:100** with Dilution Buffer
Control 1	in **200 µl** Dilution Buffer	**1:10** with Dilution Buffer
Washing Buffer		**1:20** with distilled water (e.g., add the complete contents of the flask **(50 ml)** into a graduated flask and fill with distilled water to 1000 ml).
Sample dilution: **e.g. 1:5** (Mix 50 µl Serum with 200 µl **Dilution Buffer)**		

Assay Procedure for Double Determination

Pipette	**Reagents**	**Well positions**
100 µl	Dilution Buffer	A1/2
100 µl	Standard 1 (**25 pg/ml**)	B1/2
100 µl	Standard 2 (**50 pg/ml**)	C1/2
100 µl	Standard 3 (**100 pg/ml**)	D1/2
100 µl	Standard 4 (**200 pg/ml**)	E1/2
100 µl	Standard 5 (**400 pg/ml**)	F1/2
100 µl	Standard 6 (**800 pg/ml**)	G1/2
100 µl	Standard 7 **(1600 pg/ml)**	H1/2
100 µl	Control 1	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	each well
100 µl	1:100 diluted Biotin conjugate	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	each well
100 µl	1:100 diluted HRP conjugate	each well
	Incubation: 30 min at RT, ≥350 upm	
3x 250 µl	Aspirate the contents of the wells and **wash** 3x with 250 µl Wash Buffer	each well
100 µl	Chromogenic substrate	each well
	Incubation: 30 min in the **dark** at RT	
100 µl	Stopping Solution	each well
Measure the absorbance within 15 min at **450 nm** with **≥590 nm** as reference wavelength.		

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