human Leptin ELISA

Enzyme-Immunoassay for Quantitative Determination of

human Leptin (Obese Protein)

Product-Code: E07
(96 determinations)

CE

DE/CA40/00809/17

For In-Vitro Use Only !
In the USA: For Research Use Only!

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TECHNICAL FEATURES

- Measures total Leptin concentration in **serum, plasma and other body fluids**
- Calibrated against the **WHO** International Standard: **NIBSC Code 97/594**
- Sandwich ELISA with two monoclonal antibodies in standardised 96 format **(12 x 8 wells, separately breakable)**
- Easy handling:
  - single standards with **1; 10; 25; 50 and 100 ng/ml** are supplied within our kit
  - Total Incubation time only **1.75** hours
  - lyophylised Standards, ready for use Antibody Conjugate
  - No radioactivity
- Small serum volumina sufficient

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).

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. (37). 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 (38,39) and an age dependence was shown in children and adolescents (40). 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 (20,26,37,38,41,42). 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 new 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.

INTENDED USE

The widespread importance makes leptin an interesting parameter for physicians dealing with **metabolic syndrome**, **obesity**, **cachexia** and other **metabolic disturbances**, as **diabetologists, endocrinologists**, **gynaecologists**, **andrologists**, and **psychiatrists** treating patients with **eating disorders**.

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.

Under conditions of normal eating cycles, measurement in a single blood sample collected in the morning or early afternoon is sufficient.

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.

Due to its high correlation with body fat mass leptin measurements under standardized conditions may be used as a simple and inexpensive test for determination of **body fat**.

METHODOLOGY

Assay Characteristics and Validation

The ELISA for human Leptin E07 utilizes two specific, high affinity monoclonal antibodies. It recognizes human leptin quantitatively. This assay is specific for human leptin, only low degree of cross reactions was found with mouse, rat, horse, sheep and chicken-leptin. No cross-reactivity was found with other proteins such as insulin or IGF-I.

Standards are prepared of recombinant human leptin in concentrations between **1, 10, 25, 50 and 100 ng/ml.**

The theoretical **sensitivity** of the assay yields **0.2 ng/ml** (2x SD of zero standards)

Inter-assay and intra-assay variation coefficients were both found to be less than 10% respectively. Sample dilution was found to be linear over the standard range.
Exemplary determinations are shown in the tables 1 and 2.

Table 1 : Inter-Assay-Variation: (results of 11 different sera)

	Mean value (ng/ml)	Standard deviation (ng/ml)	VC (%)
Sample 1	3.95	0.32	8.3
Sample 2	6.51	0.44	6.8
Sample 3	33.16	2.56	7.7

Table 2: Intra-Assay-Variation

	Number of determinations	Mean value (ng/ml)	Standard deviation (ng/ml)	VC (%)
Sample 1	15	4.97	0.27	5.5
Sample 2	15	37.11	2.55	6.9

The **recovery** of recombinant hLeptin in different human sera yielded on **average 102%** of the theoretical expected value.

Samples: Applicability, Preparation and Storage

Serum as well as **plasma samples** are suitable (significant deviation of hLeptin levels in corresponding Serum, Heparin- or EDTA-Plasma samples were not found). Common cell culture medium was found to

be suitable. An external sample preparation prior to assay is not required.
Samples should be handled as recommended in general: as fast as possible and chilled as soon as possible. In case there will be a longer period between the sample withdrawal and determination store the undiluted samples frozen at –20°C or below in tightly closable plastic tubes. Avoid on principal repeated freeze-thaw cycles of serum/plasma (if required, please subaliquote) although Leptin levels were found to be unaffected by few cycles (5x) in our experiments.

The high sensitivity of the assay allows measurement of hLeptin in small sample volumes. Because of the wide effective range of this ELISA kit a preparative sample dilution is generally not necessary. For most of the determinations (serum or plasma samples, and no extreme values expected) **the use of undiluted samples 20 µl per well**, **should be appropriate.** In case Leptin levels of more than 100 ng/ml are expected, the sample should and can be diluted, e.g. 1:2.

If great importance is attached to higher precision, the samples can **alternatively be diluted external on before 1:6 in Dilution Buffer VP**. After mixing use 120 µl by this way diluted sample in the assay. This alternative method reduces the number of single pipeting steps to the wells and the associated variance.

The hLeptin concentrations may be completely different in body fluids of human origin other than serum or cell culture supernatants.

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 antibody against leptin, packed in a laminate bag.

2) **Standards A-E,** lyophilized: Contain recombinant Leptin and have to be reconstituted with **250 µl Dilution Buffer VP** each. The standards should be stored after reconstitution at –20°C. Repeated freezing and thawing cycles should be avoided. Standard values are between 1 – 100 ng/ml (1; 10; 25; 50; 100 ng/ml) recombinant human leptin.

3) **Control Serum KS,** lyophilized: Contains human serum. Reconstitute with **250 µl Dilution Buffer VP.** Exact human leptin concentration and the **acceptable range** are given on the vial label. Store at -20°C after reconstitution.

4) **Dilution Buffer VP,** 15 ml, ready for use**.**

5) **anti-Leptin Antibody POD-Conjugate AK,** 12 ml, ready for use: Contains a mix of biotinylated anti-human leptin antibody and Horseradish peroxides conjugated streptavidin. Use 100 µl per well in the assay.

6) **Washing Buffer WP,** 50 ml, 20-fold concentrated solution; Washing Buffer has to be **diluted 1:20** with distilled or demineralised water before use (e.g. add the complete contents of the flask (50 ml) into a graduated flask and fill up with A.dest. to 1000 ml). Attention: After dilution the **Washing Buffer** is only limited stable, please keep in cool place.

8) **Substrate solution S,** 12 ml**,** ready for use: Horseradish-peroxides- (HRP)-substrate, stabilised H_2O_2-tetramethyl-encoding.

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, self-adhesive.

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 (recommended because of the potential danger of infection by human samples)
- Microtiterplate washer and shaker (recommended)
- Microplate reader ("ELISA-Reader") with filter for 450/620nm wavelength
- Foil welding device for laminate bags (recommended)

TECHNICAL RECOMMENDATIONS

In conducting the assay, follow strictly the test protocol.

Reagents with different lot numbers should not be mixed.

The microtiter plate and all reagents are stable until the expiry date if stored in the dark at 2-8°C (s. label).

The kit **Dilution Buffer VP** should be used for the **reconstitution** of the lyophilized components **(Standards A - E** and **Control Serum KS)**.

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.

The **shelf life** of the components **after opening** is not affected, if used appropriately. Store the unused seal stripes of the microtiter plate together with the desiccant at 2-8°C.

Reconstituted components (**Standards A – E** and **Control Serum KS**) should be stored at 2-8°C for up to 1 week. If longer storage time is needed, store the components frozen at -20°C or below. Freezing extends the expiry at least 3 months. Avoid repeated freeze-thaw

cycles. In case you plan to perform multiple independent Leptin determinations over a longer period with one kit, you should aliquot the components prior to freezing into suitable smaller volumes. This is strongly recommended.

The 1:20 diluted **Washing Buffer WP** is stable only limited. Please dilute only according to requirements.

Before use, all kit components should be brought **to room temperature**. Precipitates in buffers should in case be dissolved before use thorough mixing and warming.

Room temperature incubation means: incubation at 20 - 25°C.

The **Substrate Solution S**, stabilised H_2O_2-tetramethylbencidine, is photosensitive – store and incubate in the dark.

When performing the assay, the **Standards** (**A-E**), **Control Serum** (**KS**) and the samples should be pipette as fast as possible (e.g., 15 minutes). To avoid distortions due to differences in incubation times, the **Antibody Conjugate AK** as well as the following **Substrate Solution 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 the Substrate Solution.

PRECAUTIONS

All reagents are for in vitro use only!

The acquisition, possession and use of the kit are subjects to the regulations of the national regulatory authorities.

Reagents contain as preservative Thimerosal, however, highly diluted (0.02%). Thimerosal is toxic when swallowed and it involves a certain

danger of cumulative effects (R-Phrases 26/27/28-33-50/53 and S 13-28.1-36-45-60-61).

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 SL** provided is an acid solution! Avoid direct contact. Wear eye, hand, face and clothing protection when using this material.

The handling of potentially infectious human material (in the test kit only the provided **Control Serum KS**, has been shown negative for HBsAg, anti-HIV-1 and –2 and the individual samples) 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.

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 and Control should be assayed in duplicate.

1) **Please pipette on before in all needed wells 100 µl of Dilution Buffer**.
2) Add **20 µl** Dilution Buffer **VP** in positions A1/A2 (blank).
3) Pipette in positions B1/2 **20 µl** each **Standard A (1 ng/ml)**,
   pipette in positions C1/2 **20 µl** each **Standard B (10 ng/ml)**,
   pipette in positions D1/2 **20 µl** each **Standard C (25 ng/ml)**,
   pipette in positions E1/2 **20 µl** each **Standard D (50 ng/ml)**,
   pipette in positions F1/2 **20 µl** each **Standard E (100 ng/ml)**,
   20 µl each of the undiluted (or in the same dilution ratio as the sample) **Control KS** should be pipetted into wells G1/2.
   20 µl each of the undiluted **Samples** can be pipetted in the rest of the wells, according to requirements.
4) Cover the wells with sealing tape and incubate the plate for **1 hour**, shake with ≥ **350 rpm**.
5) 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
6) Following the last washing step, pipette **100 µl** of the **Antibody-POD-Conjugate AK** in each well and incubate **30 minutes at room temperature**, shake with ≥ **350 rpm**.
7) After incubation wash the wells **3 times** with **Washing Buffer WP** as described above, step 4.
8) Pipette **100 µl** of the **TMB-Substrate Solution S** in each well, incubate the plate for **15 minutes** in the dark.
9) Stop the reaction by adding **100 µl** of **Stopping Solution SL** in each well.
10) **Measure** the absorbance **within 30 minutes** at **450 nm (reference filter >590 nm, e.g. 620 nm).**

EVALUATION

ESTABLISIHING THE STANDARDKURVE

For the evaluation of the assay it is preconditioned that the absorbance values of the blank should be below 0.25, these of standard E should exceed 1,0. Samples, which yield higher absorbance values as **Standard E**, are beyond the standard curve, for reliable determinations these samples should be tested anew with a higher dilution.

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

Standard	**A**	**B**	**C**	**D**	**E**
ng/ml	1	10	25	50	100

1) Calculate the mean absorbance (MA) value for the blank from the duplicated determination (well A1/A2).
2) Subtract the mean absorbance (MA) of the blank from the mean absorbencies 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 standard curve should be done by using a computer programme, because the curve is in general (without respective transformation) not ideally described by linear regression. **Non-linear regression**, a **higher-grade polynomial** or **four parametric logistic (4PL) lin-log** curve fit are suitable for the evaluation. By using the corrected value of the blank as described in 2) the intersections of axis can be used additionally as a point on the standard curve (0;0), however, without constraining the curve through the zero point.

5) The Leptin concentration of the undiluted samples is achieved in ng/ml. The leptin concentration of diluted samples can be calculated by multiplication of each determined Leptin quantity with the respective dilution factor.

EXPECTED VALUES

Serum leptin levels are mainly determined by body fat mass with low levels in lean individuals and high levels in obese subjects. In addition, there is a clear gender difference with higher levels in females at a given percentage body fat. Further, leptin levels are influenced by pubertal development. Any attempt, therefore, to give ranges of expected leptin levels must account for these relationships.
Various methods for the estimation of body fat are available such as calculation of body mass index (weight (kg) divided by the square of height (m)) (BMI), bioelectric impedance assessment (BIA) or total body dual energy x-ray absorptiometry (DXA). Although the accuracy of BMI with respect to reflecting true fat mass is inferior to other more sophisticated methods such as BIA or DXA, BMI provides a number of advantages:

1) It is independent of the regression models applied.
2) It is easy to determine, only weight and height measurements are required.
3) It is retrospectively mostly available.
4) It is the most precise measure during short-term changes of fat mass, e.g. during fasting.

Therefore, the following expectation ranges of serum leptin levels were referred to BMI as the major confounding independent variable and were stratified according to gender and pubertal development (45; see figures 1-8 and tables 4 - 11). After the age of 20 years, no significant age dependence was observed. These gender and age adjusted

expectation ranges may be used to compare a measured leptin level at a given BMI with normal subjects to detect pathologic deviations.
The best-fit regression lines for the various subgroups are exponential curves of the form leptin = $a \cdot e^{(b \cdot BMI)}$.

The 5th and 95th percentiles are given by the following equations:
leptin = $a \cdot e^{(b \cdot BMI-c)}$ and leptin = $a \cdot e^{(b \cdot BMI+c)}$ respectively.

In a semi-logarithmic plot (y-axis = log leptin), these curves give straight lines. The values for a, b and c are given in table 3 according to gender and pubertal stage and also for adults. Using these values, the expectation ranges of leptin levels can be easily extended to lower or higher BMI ranges if required.

Example:
The 50th percentile for boys at Tanner stages 3 and 4 is given by the following curve:
leptin = $0.0181 \cdot e^{(0{,}2067 \,.\, BMI)}$
The 5th percentile is given by: $leptin = 0.0181 \cdot . e^{(0{,}2067 \,.\, BMI - 1{,}1919)}$
and the 95th percentile is given by: $leptin = 0.0181 \cdot e^{(0{,}2067 \,.\, BMI + 1{,}1919)}$

In a semi-logarithmic plot, these lines are parallel with an equal distance to the 50th percentile.

Calculation of standard deviation scores (SDS; Z-scores)

A convenient method to detect any deviation of a measured leptin level from the corresponding reference range is to calculate its standard deviation score by relating the leptin level at the patient's BMI to the average leptin value of the corresponding sex and age group and expressing its deviation by the x-fold standard deviation. This method may be considered as normalization to the normal reference cohort. Thus, the leptin values can be adjusted for BMI, gender and pubertal

stage/age (i.e., the influence of gender, age and BMI are removed) and may be pooled for further analysis.

Accounting for the logarithmic distribution of leptin levels, the leptin SDS can be calculated by the following equation:

leptin SDS = (ln(leptin) - ln(a) - b·BMI) / d

In this equation, ln represents the natural logarithm (referring to the basis e). The constants a, b and d are given in table 3 according to gender and pubertal stage/age.

Example:

A boy at Tanner stage 3, BMI = 25 kg/m^2, measured leptin concentration = 5 ng/ml.

leptin SDS = (ln(5) - ln (0.0181) - 0.2067·25) / 0.6850 = (1.6094 - (-4.0118) - 5.1675) / 0.6850 = 0.66

Estimation of optimal dilution of samples

Because serum leptin levels vary widely over several orders of magnitude, depending mainly on the body fat mass, adequate dilution might be a prerequisite for precise measurement. Therefore, samples should be diluted such, that the leptin concentration is near this value in order to take maximum advantage of the precision of the kit. The reference ranges provide a useful tool for a good estimation of the expected leptin value according to BMI, gender and age.

Example:

Adult woman, BMI = 45 kg/m2 (130 kgs,1.70 m height). From the reference range for adult women, the average leptin level at a BMI of 45 kg/m^2 is approximately 224 ng/ml. The optimal dilution would be 1:4.

Table 3: Constants a, b, c and d for calculation of leptin reference ranges and leptin SDS based on BMI. Groups of normal healthy individuals were stratified according to gender and pubertal stage/age. TS= Tanner stage, n= number of subjects, a,b,c, and d = constants as defined in the text.

Cohort	n	a	b	c	d
Males:					
TS 1&2	136	0.0146	0.2706	0.8821	0.5379
TS 3&4	50	0.0181	0.2067	1.1919	0.6850
TS 5	112	0.0316	0.1462	1.0821	0.6558
Adults	380	0.0130	0.2200	1.1053	0.6740
Females					
TS 1&2	136	0.0422	0.2499	0.7849	0.4786
TS 3&4	43	0.0543	0.2357	0.5745	0.3379
TS 5	157	0.2550	0.1508	0.7053	0.4301
Adults	587	0.3042	0.1467	0.8548	0.5212

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.22	0.30	0.66	1.45	1.99
12	0.28	0.39	0.85	1.86	2.56
13	0.36	0.50	1.09	2.38	3.29
14	0.46	0.64	1.40	3.06	4.22
15	0.60	0.82	1.79	3.93	5.42
16	0.76	1.05	2.30	5.04	6.96
17	0.98	1.35	2.95	6.47	8.93
18	1.25	1.73	3.79	8.31	11.5
19	1.61	2.22	4.87	10.7	14.7
20	2.07	2.85	6.25	13.7	18.9
21	2.65	3.66	8.03	17.6	24.3
22	3.41	4.70	10.3	22.6	31.2
23	4.37	6.03	13.2	29.0	40.0
24	5.62	7.75	17.0	37.2	51.4
25	7.21	9.95	21.8	47.8	65.9
26	9.26	12.8	28.0	61.4	84.7
27	11.9	16.4	35.9	78.8	109.0
28	15.3	21.1	46.1	101.0	140.0
29	19.6	27.0	59.2	130.0	
30	15.2	34.7	76.1		
31	32.3	44.6	97.7		
32	41.5	57.2	125.0		
33	53.2	73.4			
34	68.4	94.3			
35	87.8	121.0			
36	113				
37	145				

Table 4: Girls Tanner stages 1 and 2.

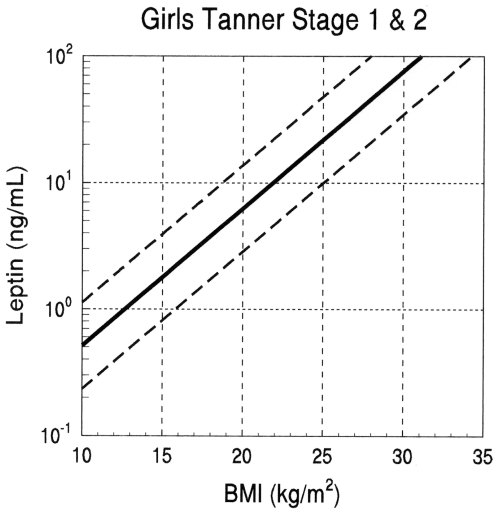

Figure 1: Reference ranges of human serum levels referring to BMI: GirlsTanner stage 1 & 2 (see text for details).

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.08	0.12	0.29	0.69	0.99
12	0.01	0.16	0.38	0.91	1.30
13	0.14	0.20	0.49	1.19	1.71
14	0.19	0.26	0.65	1.56	2.24
15	0.24	0.35	0.85	2.04	2.93
16	0.32	0.46	1.11	2.68	3.84
17	0.41	0.60	1.45	3.51	5.04
18	0.55	0.79	1.90	4.60	6.60
19	0.72	1.03	2.50	6.03	8.66
20	0.94	1.35	3.27	7.90	11.3
21	1.24	1.77	4.29	10.4	14.9
22	1.62	2.33	5.62	13.6	19.5
23	2.12	3.05	7.37	17.8	25.5
24	2.78	3.99	9.66	23.3	33.5
25	3.65	5.24	12.7	30.6	43.9
26	7.78	6.87	16.9	40.1	57.5
27	6.27	9.0	21.7	52.5	75.4
28	8.22	11.8	28.5	68.9	98.8
29	10.7	15.5	37.4	90.3	129.0
30	14.1	20.3	48.9	118.0	
31	18.5	26.6	64.2		
32	24.3	34.8	84.1		
33	31.8	45.6	110.0		
34	41.7	59.8	144.0		
35	54.6	78.4			
36	71.6	102.0			
37	93.9	134.0			
38	123.0				

Table 5: Boys Tanner stages 1 and 2.

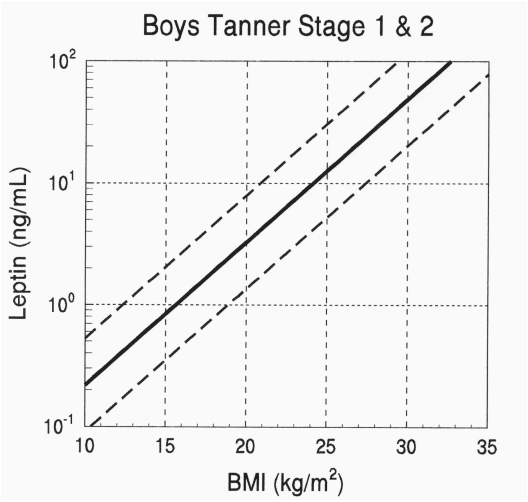

Figure 2: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 1 & 2 (see text for details).

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.32	0.41	0.73	1.29	1.63
12	0.41	0.52	0.92	1.63	2.06
13	0.52	0.66	1.16	2.07	2.61
14	0.65	0.83	1.47	2.61	3.31
15	0.83	1.05	1.87	3.31	4.19
16	1.05	1.33	2.36	4.19	5.30
17	1.33	1.68	2.99	5.30	6.71
18	1.68	2.13	3.78	6.71	8.49
19	2.13	2.69	4.79	8.5	10.8
20	2.69	3.41	6.06	10.7	13.6
21	3.41	4.31	7.67	13.61	17.2
22	4.32	5.46	9.71	17.2	21.8
23	5.46	6.91	12.3	21.8	27.6
24	6.91	8.75	15.6	27.6	34.9
25	8.75	11.1	19.7	34.9	44.2
26	11.1	14.0	24.9	44.2	56.0
27	14.0	17.7	31.6	56.0	70.9
28	17.8	22.5	39.9	70.9	89.7
29	22.5	28.4	50.5	89.7	114.0
30	28.4	36.0	63.9	114.0	144.0
31	36.0	45.6	80.9	144.0	
32	45.6	57.7	80.2	144.0	
33	57.7	73.0	102.0		
34	73.0	92.4	130.0		
35	92.4	117.0			
36	117.0	148.0			
37	148.0				

Table 6: Girls Tanner stages 3 and 4.

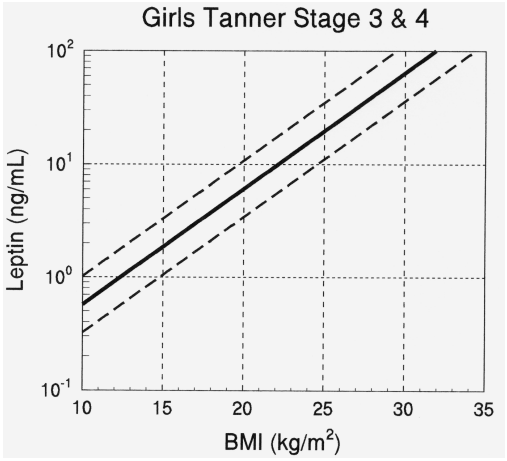

Figure 3: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 3 & 4 (see text for details).

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.03	0.05	0.18	0.58	0.94
12	0.04	0.07	0.22	0.71	1.16
13	0.49	0.08	0.27	0.88	1.43
14	0.06	0.10	0.33	1.08	1.75
15	0.07	0.12	0.40	1.32	2.16
16	0.09	0.15	0.49	1.63	2.65
17	0.11	0.18	0.61	2.00	3.26
18	0.14	0.23	0.75	2.46	4.01
19	0.17	0.28	0.92	3.03	4.93
20	0.21	0.34	1.13	3.72	6.06
21	0.26	0.42	1.39	4.58	7.46
22	0.32	0.52	1.71	5.63	9.17
23	0.39	0.64	2.10	6.92	11.3
24	0.48	0.78	2.58	8.51	13.9
25	0.59	0.96	3.18	10.5	17.0
26	0.73	1.19	3.91	12.9	21.0
27	0.89	1.46	4.80	15.8	25.8
28	1.10	1.79	5.90	19.4	31.7
29	1.35	2.20	7.26	23.9	39.0
30	1.66	2.71	8.93	29.4	48.0
31	2.05	3.33	11.0	36.2	58.9
32	2.51	4.09	13.5	44.5	72.4
33	3.09	5.04	16.6	54.7	89.1
34	3.80	6.20	20.4	67.2	109.0
35	4.68	7.62	25.1	82.6	134.0
36	5.75	9.37	30.9	101.0	
37	7.07	11.5	37.9	124.0	
38	8.7	14.2	46.7		
39	10.7	17.4	57.4		
40	13.1	21.4	70.5		

Table 7 : Boys Tanner stage 3 & 4.

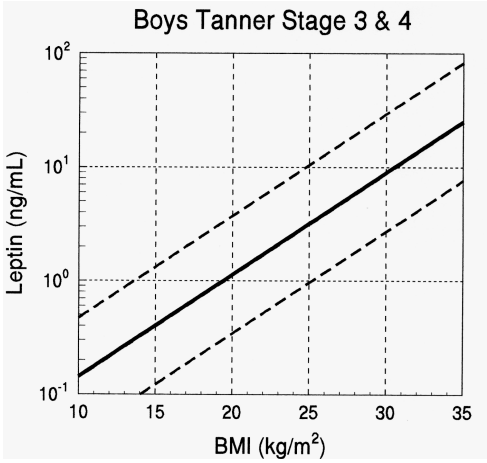

Figure 4: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 3 & 4 (see text for details).

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.50	0.66	1.34	2.71	3.62
12	0.58	0.77	1.56	3.15	4.21
13	0.67	0.89	1.81	3.67	4.89
14	0.78	1.04	2.11	4.26	5.69
15	0.91	1.21	2.45	4.96	6.62
16	1.05	1.41	2.85	5.76	7.70
17	1.22	1.64	3.31	6.70	8.95
18	1.42	1.90	3.85	7.79	10.4
19	1.66	2.21	4.48	9.06	12.1
20	1.93	2.57	5.20	10.5	14.1
21	2.24	2.99	6.05	12.3	16.4
22	2.60	3.48	7.03	14.2	19.0
23	3.03	4.04	8.18	16.6	22.1
24	3.52	4.70	9.51	19.3	25.7
25	4.09	5.46	11.0	22.4	29.9
26	4.76	6.35	12.9	26.0	34.8
27	5.53	7.39	15.0	30.3	40.4
28	6.43	8.59	17.39	35.2	47.0
29	7.48	9.99	20.2	40.9	54.7
30	8.70	11.6	23.5	47.6	63.5
31	10.1	13.5	27.3	55.3	73.9
32	11.8	15.7	31.8	64.4	85.9
33	13.7	18.3	37.0	74.9	99.9
34	15.9	21.2	43.0	87.0	116.0
35	18.5	24.7	50.0	101.0	135.0
36	21.5	28.7	58.1	118.0	
37	25.0	33.4	67.6	137.0	
38	29.1	38.8	78.6		
39	33.8	45.1	91.4		
40	39.4	52.5	106.0		

Table 8 : Girls Tanner stage 5.

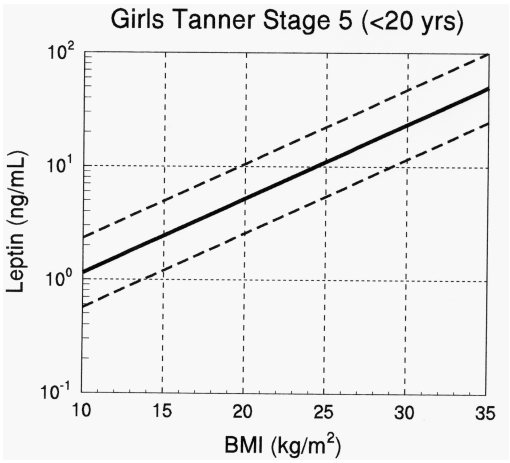

Figure 5: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 5 (see text for details).

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.03	0.05	0.16	0.47	0.73
12	0.04	0.06	0.18	0.54	0.84
13	0.05	0.07	0.21	0.62	0.97
14	0.05	0.08	0.24	0.72	1.12
15	0.06	0.10	0.28	0.84	1.30
16	0.07	0.11	0.33	0.97	1.51
17	0.08	0.13	0.38	1.12	1.74
18	0.1	0.15	0.44	1.3	2.02
19	0.11	0.17	0.51	1.50	2.34
20	0.13	0.2	0.59	1.74	2.7
21	0.15	0.23	0.68	2.01	3.13
22	0.17	0.27	0.79	2.33	3.62
23	0.20	0.31	0.91	2.69	4.19
24	0.23	0.36	1.05	3.12	4.85
25	0.27	0.41	1.22	3.61	5.62
26	0.31	0.48	1.41	4.17	6.5
27	0.36	0.55	1.63	4.83	7.52
28	0.41	0.64	1.89	5.59	8.71
29	0.48	0.74	2.19	6.47	10.1
30	0.55	0.86	2.54	7.49	11.7
31	0.64	1.00	2.94	8.67	13.5
32	0.74	1.15	3.4	10.0	15.6
33	0.86	1.33	3.94	11.6	18.1
34	0.99	1.54	4.55	13.4	20.9
35	1.15	1.79	5.27	15.6	24.2
36	1.33	2.07	6.10	18.0	28.1
37	1.54	2.39	7.06	20.8	32.5
38	1.78	2.77	8.17	24.1	37.6
39	2.06	3.21	9.46	27.9	43.5
40	2.38	3.71	10.9	32.3	50.3

Table 9 : Boys Tanner stage 5

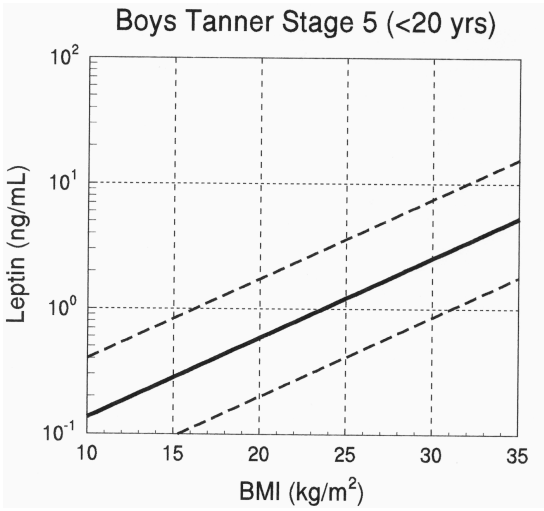

Figure 6: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 5 (see text for details).

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.46	0.65	1.53	3.59	5.10
12	0.53	0.75	1.77	4.16	5.90
13	0.61	0.87	2.05	4.82	6.83
14	0.71	1.01	2.37	5.58	7.91
15	7.82	1.17	2.75	6.46	9.17
16	0.95	1.35	3.18	7.48	10.61
17	1.10	1.57	3.68	8.66	12.3
18	1.28	1.81	4.27	10.0	14.2
19	1.48	2.10	4.94	11.6	16.5
20	1.71	2.43	5.72	13.4	19.1
21	1.99	2.82	6.62	15.6	22.1
22	2.30	3.26	7.67	18.0	25.6
23	2.66	3.78	8.88	20.9	29.3
24	3.08	4.38	10.3	24.2	34.3
25	3.57	5.07	11.9	28.0	39.7
26	4.13	5.87	13.8	32.4	46.0
27	4.79	6.79	16.0	37.5	53.3
28	5.54	7.87	18.5	43.5	61.7
29	6.42	9.11	21.4	50.4	71.5
30	7.43	10.6	24.8	58.3	82.8
31	8.61	12.2	28.7	67.5	95.8
32	9.97	14.1	33.3	78.2	111.0
33	11.5	16.4	38.5	90.5	129.0
34	13.4	19.0	44.6	105.0	149.0
35	15.5	22.0	51.6	121.0	
36	17.9	25.4	59.8	141.0	
37	20.8	29.5	69.3		
38	24.0	34.1	80.2		
39	27.8	39.5	92.9		
40	32.2	45.7	108.0		

Table 10: Adult women.

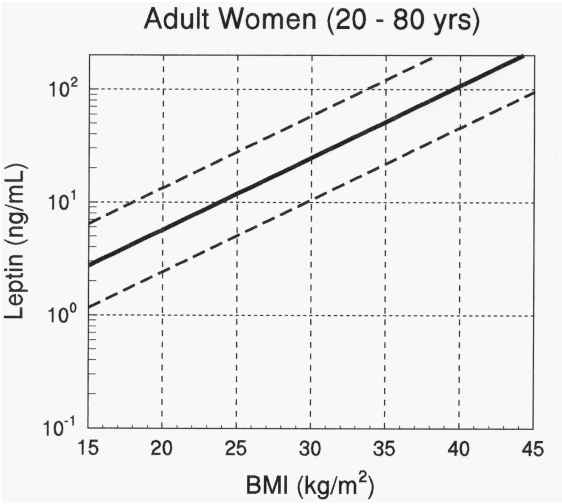

Figure 7: Reference ranges of human serum levels referring to BMI: Adult women (see text for details)

Percentile (µg/L)					
BMI (kg/m²)	1	5	50	95	99
11	0.03	0.05	0.15	0.44	0.69
12	0.04	0.06	0.18	0.55	0.87
13	0.05	0.08	0.23	0.69	1.08
14	0.06	0.09	0.28	0.85	1.34
15	0.07	0.12	0.35	1.06	1.67
16	0.09	0.15	0.44	1.33	2.09
17	0.12	0.18	0.55	1.65	2.60
18	0.14	0.23	0.68	2.06	3.24
19	0.18	0.28	0.85	2.57	4.04
20	0.22	0.35	1.06	3.20	5.03
21	0.23	0.44	1.32	3.98	6.27
22	0.35	0.54	1.64	4.97	7.81
23	0.43	0.78	2.05	6.19	9.73
24	0.54	0.85	2.55	7.71	12.1
25	0.67	1.05	3.18	9.61	15.1
26	0.83	1.31	3.96	12.0	18.8
27	1.04	1.64	4.94	14.9	23.5
28	1.30	2.04	6.15	18.6	29.2
29	1.61	2.54	7.67	23.2	36.4
30	2.01	3.16	9.56	28.9	45.4
31	2.51	3.94	11.9	36.0	56.6
32	3.12	4.91	14.8	44.9	70.5
33	3.89	6.12	18.5	55.8	87.8
34	4.85	7.63	23.0	69.6	109.0
35	6.04	9.51	28.7	86.7	136.0
36	7.53	11.8	35.8	108.0	
37	9.38	14.8	44.6	135.0	
38	11.7	18.4	55.5		
39	14.6	22.9	69.2		
40	18.2	28.6	86.2		

Table 11 Adult men.

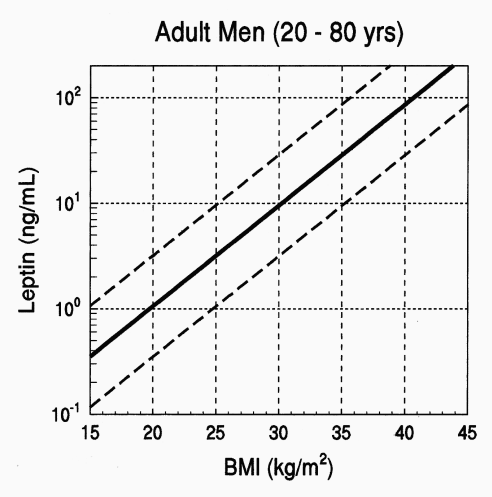

Figure 8: Reference ranges of human serum levels referring to BMI: Adult men (see text for details).

REFERENCES

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. 1994 Positional cloning of the mouse obese gene and its human homologue. Nature. 372:425-432.
2. Halaas JL, Gajiwala KS, Maffei M, et al. 1995 Weight-reducing effects of the plasma protein encoded by the obese gene. Science. 269:543-546.
3. MacDougald OA, Hwang CS, Fan H, Lane MD. 1995 Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. Proc Natl Acad Sci U S A. 92:9034-9037.
4. Rentsch J, Chiesi M. 1996 Regulation of ob gene mRNA levels in cultured adipocytes. FEBS Lett. 379:55-59.
5. Wabitsch M, Jensen PB, Blum WF, et al. 1996 Insulin and cortisol promote leptin production in cultured human fat cells. Diabetes. 45:1435-1438.
6. Stephens TW, Basinski M, Bristow PK, et al. 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature. 377:530-532.
7. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. 1995 Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science. 269:546-549.
8. Pelleymounter MA, Cullen MJ, Baker MB, et al. 1995 Effects of the obese gene product on body weight regulation in ob/ob mice. Science. 269:540-543.
9. Levin N, Nelson C, Gurney A, Vandlen R, de-Sauvage F. 1996 Decreased food intake does not completely account for adiposity reduction after ob protein infusion. Proc Natl Acad Sci U S A. 93:1726-1730.

10. Tartaglia LA, Dembski M, Weng X, et al. 1995 Identification and expression cloning of a leptin receptor, OB-R. Cell. 83:1263-1271.
11. Chen H, Charlat O, Tartaglia LA, et al. 1996 Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell. 84:491-495.
12. Lee GH, Proenca R, Montez JM, et al. 1996 Abnormal splicing of the leptin receptor in diabetic mice. Nature. 379:632-635.
13. Lynn RB, Cao GY, Considine RV, Hyde TM, Caro JF. 1996 Autoradiographic localization of leptin binding in the choroid plexus of ob/ob and db/db mice. Biochem Biophys Res Commun. 219:884-889.
14. Considine RV, Considine EL, Williams CJ, Hyde TM, Caro JF. 1996 The hypothalamic leptin receptor in humans: identification of incidental sequence polymorphisms and absence of the db/db mouse and fa/fa rat mutations. Diabetes. 45:992-994.
15. Sinha MK, Opentanova I, Ohannesian JP, et al. 1996 Evidence of free and bound leptin in human circulation. J Clin Invest. 98:1277-1282.
16. Ahima RS, Prabakaran D, Mantzoros C, et al. 1996 Role of leptin in the neuroendocrine response to fasting. Nature. 382:250-252.
17. Chehab FF, Lim ME, Lu R. 1996 Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. Nat Genet. 12:318-320.
18. Schwartz MW, Baskin DG, Bukowski TR, et al. 1996 Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. Diabetes. 45:531-535.

19. Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG. 1996 Identification of targets of leptin action in rat hypothalamus. J Clin Invest. 98:1101-1106.
20. Campfield LA, Smith FJ, Burn P. 1996 The OB protein (leptin) pathway - a link between adipose tissue mass and central neural networks. Horm Metab Res. 28:619-632.
21. Rohner-Jeanrenaud F, Cusin I, Sainsbury A, Zakrzewska KE, Jeanrenaud B. 1996 The loop system between neuropeptide Y and leptin in normal and obese rodents. Horm Metab Res. 28:642-648.
22. Chan YY, Steiner RA, Clifton DK. 1996 Regulation of hypothalamic neuropeptide-Y neurons by growth hormone in the rat. Endocrinol. 137:1319-1325.
23. Pierroz DD, Catzeflis C, Aebi 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.
24. Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. 1995 Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med. 1:1311-1314.
25. Maffei M, Halaas J, Ravussin E, et al. 1995 Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med. 1:1155-1161.
26. Considine RV, Sinha MK, Heiman ML, et al. 1996 Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med. 334:292-295.
27. Kolaczynski JW, Considine RV, Ohannesian J, et al. 1996 Responses of leptin to short-term fasting and refeeding in humans: A link with ketogenesis but not ketones themselves. Diabetes. 45:1511-1515.

28. Harris RB, Ramsay TG, Smith SR, Bruch RC. 1996 Early and late stimulation of ob mRNA expression in meal-fed and overfed rats. J Clin Invest. 97:2020-2026.
29. Kolaczynski JW, Ohannesian J, Considine RV, Marco C, Caro JF. 1996 Response of leptin to short-term and prolonged overfeeding in humans. J Clin Endocrinol Metab. 91:4162-4165.
30. Saladin R, De-Vos P, Guerre-Millo M, et al. 1995 Transient increase in obese gene expression after food intake or insulin administration. Nature. 377:527-529.
31. Cusin I, Sainsbury A, Doyle P, Rohner-Jeanrenaud F, Jeanrenaud B. 1995 The ob gene and insulin. A relationship leading to clues to the understanding of obesity. Diabetes. 44:1467-1470.
32. Kolaczynski JW, Nyce MR, Considine RV, et al. 1996 Acute and chronic effects of insulin on leptin production in humans: studies in vivo and in vitro. Diabetes. 45:699-701.
33. Malström R, Taskinen M-R, Karonen S-L, Yki-Järvinen H. 1996 Insulin increases plasma leptin concentrations in normal subjects and patients with NIDDM. Diabetologia. 39:993-996.
34. De-Vos P, Saladin R, Auwerx J, Staels B. 1995 Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. J Biol Chem. 270:15958-15961.
35. Slieker LJ, Sloop KW, Surface PL, et al. 1996 Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. J Biol Chem. 271:5301-5304.
36. Miell JP, Englaro P, Blum WF. 1996 Dexamethasone induces an acute and sustained rise in circulating leptin levels in normal human subjects. Horm Metab Res. 28:704-707.
37. Sinha MK, Ohannesian JP, Heiman ML, et al. 1996 Nocturnal rise of leptin in lean, obese, and non-insulin- dependent diabetes mellitus subjects. J Clin Invest. 97:1344-1347.

38. Havel PJ, Kasim-Karakas S, Dubuc GR, Mueller W, Phinney SD. 1996 Gender difference in plasma leptin concentrations. Nature Med. 2:949-950.

39. Rosenbaum M, Nicolson M, Hirsch J, et al. 1996 Effects of gender, body composition, and menopause on plasma concentrations of leptin. J Clin Endocrinol Metab. 81:3424-3427.

40. Hassink SG, Sheslow DV, de Lancey E, Opentanova I, Considine RV, Caro JF 1996 Serum leptin in children with obesity: relationship to gender and development. Pediatrics. 98:201-203.

41. Scholz GH, Englaro P, Thiele I, et al. 1996 Dissociation of serum leptin concentration and body fat content during long term dietary intervention in obese individuals. Horm Metab Res. 28:718-723.

42. Caro JF, Kolaczynski JW, Nyce MR, et al. 1996 Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. Lancet. 348:159-161.

43. Robinson CJ, Gaines-Das R,Woollacott D, et al. 2001 The first international standard for human leptin and the first international standard for mouse leptin: comparison of candidate preparations by in vitro bioassays and immunoassays. J Molecular Endocrinol. 27: 69-76.

44. Adresse NIBSC: Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

45. Blum WF, Juul A, Reference ranges of serum leptin, s.318-326. In: Leptin –the voice of adipose tisue, Bluum WF et al, eds.. Johann Ambrosius Verlag, Heidelberg, 1977.

SUMMARY – Mediagnost hLEPTIN ELISA E07

Reconstitution/ Dilution of reagents		
Standards A-E	reconstitute in **Dlution Buffer VP**	**250 µl**
Control Serum KS	reconstitute in **Dilution Buffer VP**	**250 µl**
Washing Buffer WP	dilute in **A. dest.** (e.g. add the complete contents of the flask (50 ml) into a graduated flask and fill with A.dest. to 1000 ml).	**1:20**
Sample dilution is in general not necessary, use **20 µl undiluted** per determination		
Before assay procedure bring all **reagents** to the **room temperature**.		

Proposal of Assay Procedure for double determinations

Pipette	**Reagents**	**Well positions**
100 µl	Dilution Buffer **VP**	**Pipette in all required number of wells**
20 µl	Dilution Buffer **VP** as blank	A1 and A2
20 µl	Standard **A** (**1 ng/ml**)	B1 and B2
20 µl	Standard **B** (**10 ng/ml**)	C1 and C2
20 µl	Standard **C** (**25 ng/ml**)	D1 and D2
20 µl	Standard **D** (**50 ng/ml**)	E1 and E2
20 µl	Standard **E** (**100 ng/ml**)	F1 and F2
20 µl	Control Serum **KS**	G1 and G2
20 µl	Sample	Pipette sample in the rest of the wells according to requirements
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 each Wash Buffer **WP**/**well**	each well
100 µl	Antibody-POD-Konjugat **AK**	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: 15 min in the **dark** at RT		
100 µl	Stop Solution **SL**	each well
Measure the absorbance within 30 min at **450 nm**.		