Manufactured for Immuno-Biological Laboratories Inc.
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Leptin ELISA
sensitive

Enzyme Immunoassay for the Precise Determination of

human Leptin

- with high sensitivity -

for Research Use Only.
not for use in diagnostic procedures.

Σ 96

REF E077

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Manufactured by Mediagnost / Germany
for IBL-America Minneapolis MN55432

Symbols

DIN EN ISO 15223-1

		Expiry date
		Consider instructions for use
LOT		Lot-Batch Number
		Manufactured by
REF		Catalogue Number
		Store at between
		Contains sufficient for x tests
		Keep away from sunlight
		Incubation time
°C		Incubate at
		Shaking
MTP		Miktrotiterplate
Rec in		Reconstitute in
SPE		Sample
AbIgG	AK IgG	Antibody-Biotin-Conjugate AK IgG
AbIgM	AK IgM	Antibody-Biotin-Conjugate AK IgM
Conj	EK	Streptavidin-HRP-Conjugate EK
DILU X	VP	Dilute in Buffer X
CAL X	STD 1/ STD 2	Standard X
WASHBUF 20x	WP	Washing Buffer Concentrate
WASHBUF		Washing Buffer
SUBST TMB	S	Substrate
H_2SO_4	SL	Stop Solution
TAPE		Cover Plate with sealing tape
MEASURE		Measure plate within 30 min at 450 nm (Reference filter ≥590nm)

For Research Use Only.
Not for use in diagnostic procedures.
CAUTION: Not for human or animal therapeutic or diagnostic use.

Table of Contents

Human Leptin **SENSITIVE** ELISA cat.-no.: E077

- For research use only!
- Measures total Leptin concentration in serum, plasma and other body fluids
- Calibrated against the WHO International Standard: NIBSC Code 97/594
- single standards with 0.05; 0.5; 1.5; 3.5 and 5 ng/ml are supplied within this kit
- Total incubation time only 1.75 hours
- 2 Control Sera for Quality Control according GLP
- ready-to-use Antibody Conjugate
- Microtiterplate seperately breakapart
- The high sensitivity (range of standards 0.05 - 5 ng/ml) allows:
    - precise measurement
    - measurement in small serum volumes
    - measurement of low leptin concentrations in specimens other than serum
    - such as urine, cerebrospinal fluid, and cell culture media

INTENDED USE

Measurement of human Leptin in human serum, plasma, urine, saliva, breast milk for research use only!

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).

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.

This ELISA kit is suited for measuring human leptin in serum or plasma, other biological fluids (e.g. urine or cerebrospinal fluid), and conditioned adipocyte culture media for scientific purposes.

REAGENTS PROVIDED

1)	MTP	**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)	STD	**Standards A-E, 1 ml** lyophilized: Contain recombinant Leptin and have to be reconstituted with **1 ml Dilution Buffer VP** each. After using store the reconstituted standards in the original flasks as soon as possible at –20°C. When using the standards anew, please thaw them rapidly but gently (no temperature rise over the room temperature and no powerful vortexing), 3 of these freezing-thawing cycles showed no influence on the assay. Standard values are between 0.05 – 5 ng/ml (0.05; 0.5; 1.5; 3.5; 5 ng/ml) recombinant human leptin
3)	BUF	**Dilution Buffer VP, 60 ml**, ready for use, please use for the **reconstitution of Standards A-E, Control Sera KS1 & KS2.**
4)	Control	**Control Sera KS1 & KS2,** each **250 µl** lyophilized: Contains human serum. Reconstitute each with **250 µl Dilution Buffer VP.** Exact human leptin concentration and the acceptable range are given on the certificate. The reconstituted Control Sera must be stored in the original flask as soon as possible at –20°C after use. When using anew, please thaw it rapidly but gently (no temperature rise over the room temperature and no powerful vortexing), 3 of these freezing-thawing cycles showed no influence on the assay.
5)	Ab CONJ	**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)	WASHBUF 20x	**Washing Buffer (WP), 50 ml**, 20 X concentrated solution. **Washing Buffer (WP)** 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 4 weeks stable, dilute only according to requirements.
7)	SUBST	**Substrate (S), 12 ml**, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbencidine.
8)	H_2SO_4	**Stopping Solution (SL), 12 ml**, ready for use, 0.2 M sulphuric acid, Caution acid!
9)		Sealing tape for covering of the microtiterplate, 2 x, adhesive.

MATERIALS REQUIRED BUT NOT PROVIDED

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

WARNINGS AND PRECAUTIONS

For research and professional use only.
Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.

Before use, all kit components should be brought **to room temperature at 20 - 25°C**. Precipitates in buffers should be dissolved before use by thorough mixing and warming. Do not mix reagents of different lots. Do not use expired reagents.

The microplate contains snap-off strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.

Caution: This kit contains material of human and/or animal origin. Source human serum for the Control Sera provided in this kit was tested by FDA recommended methods and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV) antibody. No known test methods can offer total assurance of the absence of infectious agents; therefore all components and specimens should be treated as potentially infectious.

Following components contain < 0.01% 2-Methyl-4-isothiazolin-3-one solution as preservative : **AK, VP**

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 contain < 0.01%(w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-isothiazol-3-one as preservative: **AK, VP, WP**

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 S28.1	After contact with skin, wash immediately with plenty of water

Stop solution contains 0.2 M Sulfuric 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
S28.1	After contact with skin, wash immediately with plenty of water
S36/37	Wear suitable protective clothing and gloves.

Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step. Use separate pipette tips for each sample, control and reagent to avoid cross contamination. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.

TMB-Substrate (S) contains 3,3´,5,5´ Tetramethylbenzidine. Store and Incubate in the dark.

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
S28.1	After contact with skin, wash immediately with plenty of water
S36/37	Wear suitable protective clothing and gloves

General 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.
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.

PRINCIPLE

This ELISA testkit for hLeptin measurements (cat.-no.: E077) is a so-called Sandwich-Assay using two specific and high affinity antibodies. The Leptin in the samples binds to the first antibody coated on the microtiterplate. In the following step the second specific anti-Leptin-Antibody binds in turn to the immobilised Leptin. The second antibody is biotinylated and will be applied in a mixture with a Streptavidin-Peroxidase-Enzyme Conjugate. In the closing substrate reaction the turn of the colour will be catalysed quantitatively depending on the Leptin-level of the samples.

SPECIMEN

Serum and plasma samples, urine, saliva and breast milk are applicable.

Significant deviation of hLeptin levels in corresponding serum- or EDTA plasma samples were not found. If commercially available tubes for Citrat-plasma are used for preparation, samples will be diluted, resulting Leptin values will be therefore reduced.

The blood sample for serum preparation should be gained according to standardized venipucture procedure. Hemolytic reactions have to be avoided.

The blood has to be allowed to clot 30-60 min at RT. After complete clotting, serum is separated by centrifugation (3000g, 10 min).

In case of normal nutritional status serum and plasma samples should be gained in the morning or the early afternoon, because leptin undergoes circadian changes with a maximal serum value at 2 am.

Storage of the samples

Storage at RT max. 2 days

Storage at –20°C max. 2 years

Are not allowed to have more than five freeze/thaw cycles.

Sample Preparation

Samples have to be diluted in Dilution Buffer (VP).
We recommend a standard dilution on 1:10.

<u>Suggestion for dilution protocol:</u>

Dilute e.g. 225 µl Dilution Buffer VP in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add 25 µl serum or plasma (dilution 1:10). After mixing use 2 x 100 µl from the dilution in the assay.

If Leptin levels over 50 ng/ml are expected, the sample should be diluted higher in Dilution Buffer VP, e.g. 1:20.
The hLeptin concentrations may be completely different in body fluids of human origin other than serum or cell culture supernatants. In samples of urine or breast milk very low levels were determined. Furthermore this kind of samples must be diluted at least 1:5. If samples are used undiluted, the reaction will be interfered and the values are reduced (s. Assay Characteristics and Validation).

TECHNICAL NOTES

The assay has to be conducted strictly according the test protocol herein.

Reagents with different lot numbers cannot be mixed. The microtiterplate and reagents are stable until the indicated expiry if stored unopened and protected from sunlight at 2 – 8°C.

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. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming.

Incubation at room temperature means: 20-25°C

The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiterplate 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 microtiterplate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non fuzzy absorbent tissue.

Manual washing 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 microtiterplate 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.

Standards and Controls

For the reconstitution of the lyophilised components (Standards A - E and Control Sera KS1 &KS2) the kit Dilution Buffer VP has to be used. It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam!) with a Vortex mixer.

The reconstituted standard and controls can be stored for 2 month at –20°C. Repeated freeze/thaw cycles have to be avoided.

Washing Buffer

The required volume of washing buffer is prepared by 1:20 dilution of the provided 20fold concentrate with deionised water. The diluted washing buffer is stable for 4 weeks at 2-8°C. It has to be at room temperature for usage!

Microtiterplate

Unused microtiterplate stripes have to be stored airtight together with the desiccant bag at 2-8°C. The labelled expiry is not influenced in case of proper storage.

ASSAY PROCEDURE

All determinations (Standards, Control Sera KS1 & KS2 and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

When performing the assay, the Standards, Control Sera and the samples should be pipetted as fast as possible (e.g., <15 minutes).

All incubations have to be conducted at room temperature (20-25°C)!

To avoid distortions due to differences in incubation times, Antibody-POD-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.

1) add 100 µl Dilution Buffer VP to the first wells (blank). Subsequently, add 100µL of each Standard or diluted Control (KS1&KS2) or diluted Sample to the following wells.

2) cover the wells with sealing tape and incubate the plate for 1 hour shaking with 350 rpm.

3) after incubation aspirate the contents of the wells into a disinfectant (possible theoretically 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

4) pipette 100 µl of the Antibody-POD-Conjugate AK in each well and incubate 30 minutes shaking with 350 rpm.

5) after incubation wash the wells 3 times with Washing Buffer as described in step 3

6) pipette 100 µL of the Substrate (S) in each well.

7) incubate the plate for 15 minutes in the dark at room temperature (20 - 25°C).

8) stop the reaction by adding 100 µL of Stopping Solution (SL).

9) measure the colour reaction within 30 minutes at 450 nm (reference filter ≥590 nm).

CALCULATION OF RESULTS

Establishing the Standard Curve

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 be above 1.0.

Samples, which yield higher absorbance values than Standard E are beyond the standard curve, for reliable determinations these samples should be tested anew with a higher dilution.

Standards are provided in the following concentrations (use the concentration unit as preferred):

Standard	**A**	**B**	**C**	**D**	**E**
ng/ml	0.05	0.5	1.5	3.5	5

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. 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 concentration in ng/ml of the samples can be calculated by multiplication with the respective dilution factor.

The exemplary shown standard curve in Fig.1 cannot be used for calculation of your test results. You have to establish a standard curve for each test you conduct!

Exemplary calculation of the leptin concentration of an undiluted sample:

Measured extinction of your sample 0.293

Measured extinction of the blank 0.015

Your measurement programm will calculate the leptin concentration of the diluted sample automatically by using the difference of sample and blank (0.278) for the calculation. You only have to determine the most suitable curve fit (here: polynomial 2nd degree).

In this exemplary case the following equation is solved by the program to calculate the Leptin concentration in the sample:

$$0.278 = -0.0089658+0,64743x -0.034025x^2$$
$$0.4524 = x$$

if the dilution factor (1:10) is taken into account the leptin concentration of the undiluted sample is

0.4524 x 10 ng/mL=4.4524 ng/mL

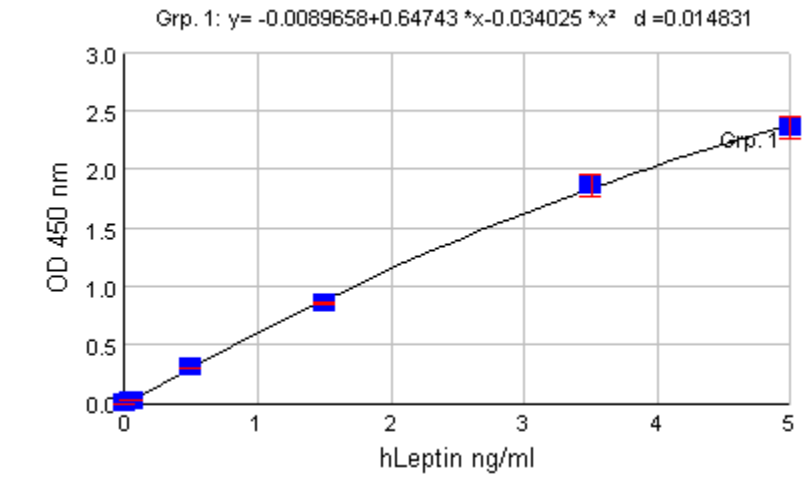

Fig. 1: Exemplary Standard Curve with a polynomial 2rd degree as curve fit.

PERFORMANCE CHARACTERISTICS

Standards

The Standards of the ELISA E077 are prepared from recombinant Leptin in concentrations of 0.05; 0.5; 1.5; 3.5; and 5 ng/ml.

Sensitivity

The analytical sensitivity of the assay yields 0.01 ng/ml (2x SD of zero standards in 12-fold determinations).

Specificity

This assay is specific for human leptin. Sera of the cited species were used as sample in this assay system. No measurable cross reactivity was detected in serum of:

Horse, Cow, Chicken, Rabbit, Dog, Guinea pig, Sheep, Mouse, Goat, Donkey, Rat, Cat, Pig

Reproducibility and Precision

The inter- and intra assay coefficients of variability are below 10%. Exemplary determinations are shown in table 1 and 2.

Table 1 : Inter-Assay-Variation

	Mean value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	**2.04**	**0.147**	**7.2**
Sample 2	**6.93**	**0.423**	**6.1**
Sample 3	**14.86**	**1.11**	**7.5**

Table 2: Intra-Assay-Variation

	Mean value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	**22.44**	**1.28**	**4.35**
Sample 2	**4.1**	**0.108**	**2.63**

EXEMPLARY 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.
The following exemplary 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 2-9 and tables 6 - 13). After the age of 20 years, no significant age dependence was observed. These. The best-fit regression lines for the various subgroups are exponential curves of the form:

$$Leptin = a \cdot e^{(b \cdot BMI)}$$

Appendix /Anhang

Table 3: Constants a, b, c and d for calculation of leptin 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 (see Chapter expected Normal Values).

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

Table 4: Girls Tanner stages 1 and 2

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.		
33	53.2	73.4			
34	68.4	94.3			
35	87.8	121.			
36	113				
37	145				

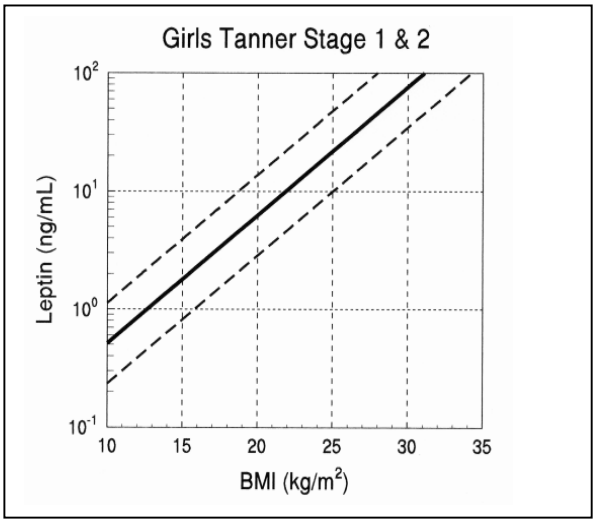

Figure 2: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 1 & 2 (see text for details)

Table 5: Boys Tanner stages 1 and 2

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				

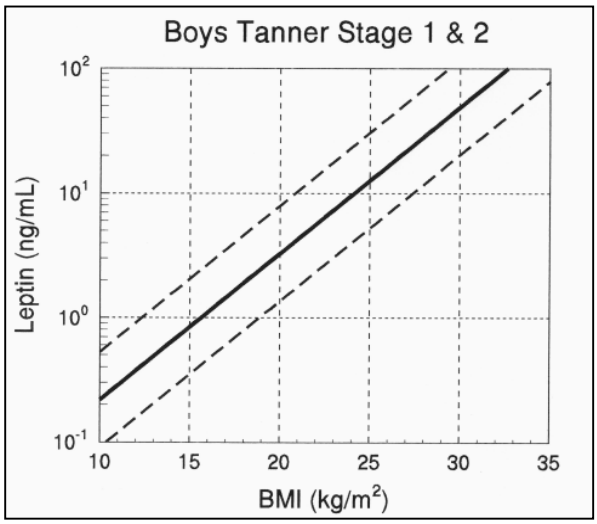

Figure 3: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 1 & 2 (see text for details)

Table 6: Girls Tanner stages 3 and 4.

Percentile (µg/L)					
BMI	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				

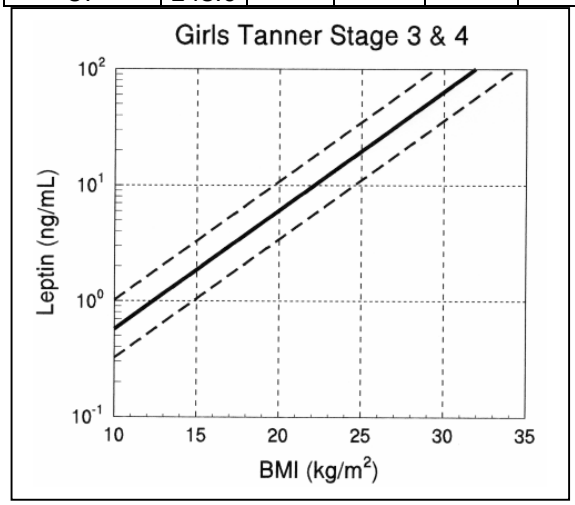

Figure 4: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 3 & 4 (see text for details).

Table 7 : Boys Tanner stage 3 & 4

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.	124.0	
38	8.7	14.2	46.7		
39	10.7	17.4	57.4		
40	13.1	21.4	70.5		

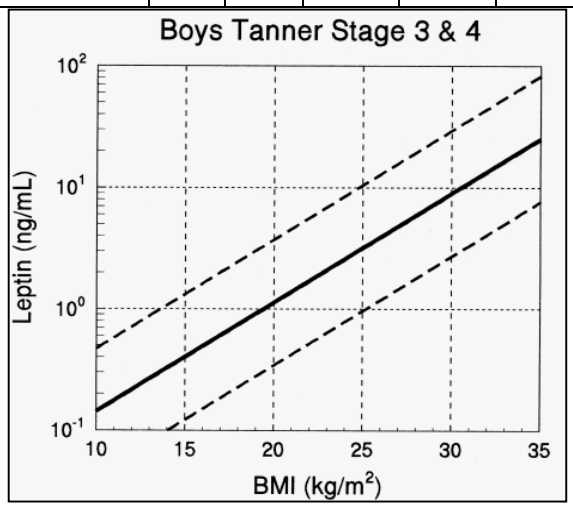

Figure 5: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 3 & 4 (see text for details).

Table 8 : Girls Tanner stage 5.

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		

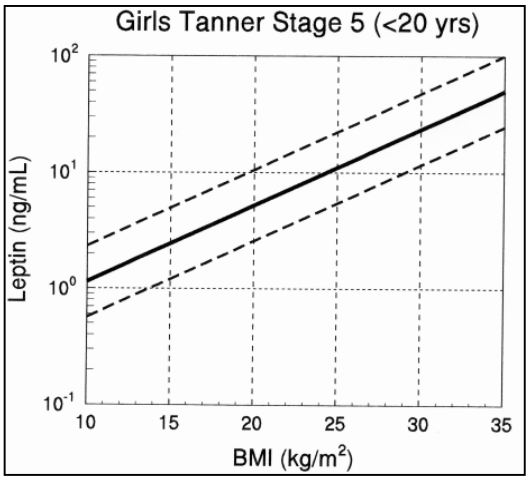

Figure 6: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 5 (see text for details)

Table 9 : Boys Tanner stage 5

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

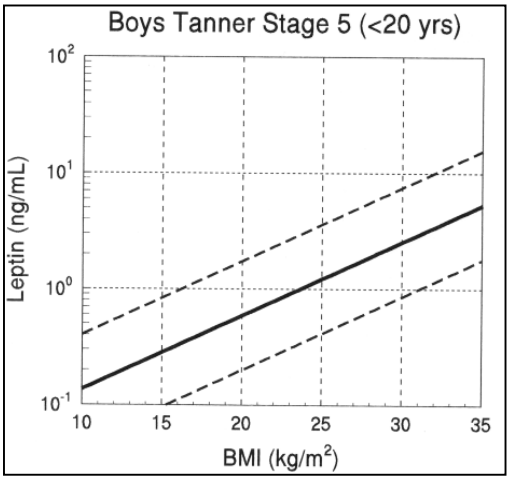

Figure 7: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 5 (see text for details).

Table 10: Adult women.

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		

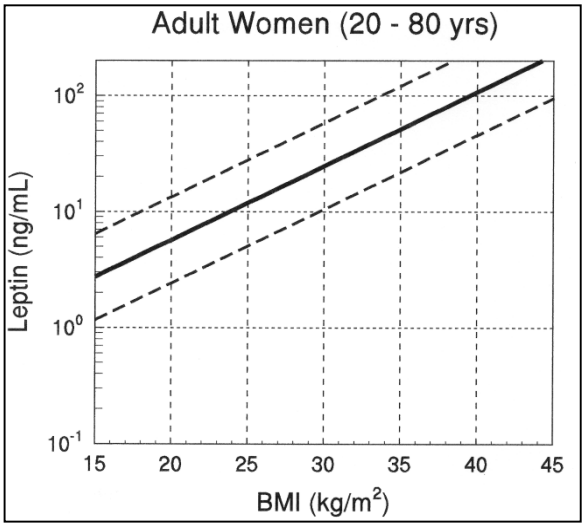

Figure 8: Reference ranges of human serum levels referring to BMI: Adult women (see text for details)

Table 11 Adult men

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		

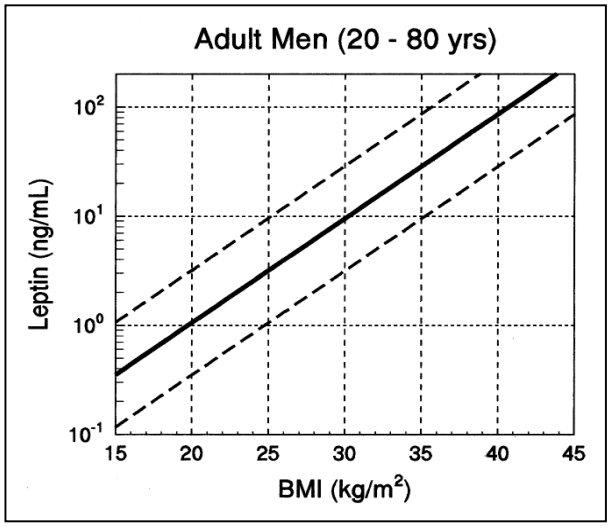

Figure 9: Reference ranges of human serum levels referring to BMI: Adult men (see text for details).

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SUMMARY –hLEPTIN-SENSITIVE ELISA E077

Reconstitution/ Dilution of reagents		
Standards A-E	reconstitute in **Dlution Buffer VP** (after using, store at –20°C)	**1 ml**
Control Serum KS1&KS2	reconstitute in **Dilution Buffer VP** (after using, store at –20°C)	**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 varies regarding the sample type, serum samples generally e.g. 1:10, use 100 µl 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** as blank	A1 and A2
100 µl	Standard **A** (**0.05 ng/ml**)	B1 and B2
100 µl	Standard **B** (**0.5 ng/ml**)	C1 and C2
100 µl	Standard **C** (**1.5 ng/ml**)	D1 and D2
100 µl	Standard **D** (**3.5 ng/ml**)	E1 and E2
100 µl	Standard **E** (**5 ng/ml**)	F1 and F2
100 µl	Control Serum **KS1**	G1 and G2
100 µl	Control Serum **KS2**	H1 and H2
100 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 **(20-25°C)**, 350 rpm

3x 300 µl	Aspirate the contents of the wells and **wash** 3x with **300 µl** Wash Buffer **WP/well**	each well
100 µl	Antibody-HRP-Conjugate **AK**	each well

Incubation: 30 min at RT **(20-25°C)**, 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: 15 min in the **dark** at RT **(20-25°C)**

100 µl	Stop Solution **SL**	each well
Measure the absorbance within 30 min at **450 nm** (≥ 590 nm reference)		

REF E077 **International Test description**

STD **A-E**	**A -E**	**Rec in** 1ml **BUF** **VP**	
Control	**KS1 & KS2**	**Rec in** 250 µl **BUF** **VP**	**1:10** **DILU** **BUF** **VP**
WASHBUF **20x**	**WP**		**1:20** **DILU** **A. dest.**

SPE	**1:10** **DILU** **BUF** **VP**
°C **20-25 °C**	

100 µl	**BUF** **VP**	A1/2
100 µl	**STD** **A** **(0.05 ng/ml)**	B1/2
100 µl	**STD** **B** **(0.5 ng/ml)**	C1/2
100 µl	**STD** **C** **(1.5 ng/ml)**	D1/2
100 µl	**STD** **D** **(3.5 ng/ml)**	E1/2
100 µl	**STD** **E** **(5 ng/ml)**	F1/2
100 µl	**CONTROL** **KS1** **1:10** **DILU** **BUF** **VP**	G1/2
100 µl	**CONTROL** **KS2** **1:10** **DILU** **BUF** **VP**	H1/2
100 µl	**SPE** **1:10** **DILU** **BUF** **VP**	
TAPE		

1 h **°C** **20-25** ↔ **350 rpm**

3x 300 µl	**3x** **WASHBUF** **WP**
100 µl	**AbCONJ** **AK**
TAPE	

0.5 h **°C** **20-25** ↔ **350 rpm**

3x 300 µl	**3x** **WASHBUF** **WP**
100 µl	**SUBST** **TMB** **S**

15 min **°C** **20-25**

100 µl	**H_2SO_4** **SL**
MEASURE	