Product information

Useŕs Manual

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Leptin ELISA

Enzyme Immunoassay for quantitative determination of human Leptin

CE

IVD

REF **DEE007**

Σ **96 wells**

Version 14-01/16
DMC
Updated 160503

ENGLISH Instructions for use

Leptin ELISA DEE007	**96 Determinations**
Principle of the test	Enzyme-linked Immunoassay
Duration (incubation period)	1.75 h
Antibodies	monoclonal antibodies
Buffer	Ready for use and 20fold concentrate
Standard	5 single standards: 1 – 100 µg/L, recombinant human Leptin
Reference material	International Standard WHO/ NIBSC 97/594, recombinant Leptin
Assay Range	0.25 – 100 µg/L
Control	2 control sera, freeze-dried
Sample	human serum / plasma
Required sample volume	20 µL or 40 µL for single or duplicate measurement
Sample dilution	undiluted
Analytical sensitivity	≤ 0.25 µg/L
Intra- / Interassay Variance	<15 %
Reference values	Blum WF, Juul A; Reference ranges of serum leptin, In: Leptin- the voice of adipose tissue, Blum WF et al. eds. Johann Ambrosius Verlag, Heidelberg, 1997

1 INTENDED USE

The ELISA DEE007 is intended to be used for quantitative measurement of human Leptin in human serum and plasma samples.

2 INTRODUCTION

Leptin, the product of the ob gene (1,2), is a 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. 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 by 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.
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.
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.

3 ASSAY PRINCIPLE

The Demeditec ELISA for Leptin DEE007 is a so-called Sandwich-Assay using two specific and highly affine antibodies. The Leptin in the samples binds to the first antibody coated on the microtiter plate. 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 subsequent substrate reaction the turn of the colour will be catalysed quantitatively depending on the Leptin-level of the samples.

4 WARNINGS AND PRECAUTIONS

For In Vitro Diagnostic Use only. For Professional use only.
The Demeditec kit is suitable only for in vitro diagnostics and not for internal use in humans and animals. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Demeditec will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided. A Material Safety Data sheet is available on request.

Do not use obviously damaged or microbial contaminated or spilled material.

Caution: This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.

Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.

Human Serum
Following components contain human serum: **Control Sera KS1 and KS2, and Standards A-E**
Source human serum for the control sera provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.

Reagents A-E, AK, VP, WP
Contain as preservative a mixture of **5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one** (<0.015%)

H317	May cause an allergic skin reaction.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P272	Contaminated work clothing should not be allowed out of the workplace.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P333+P313	If skin irritation or rash occurs: Get medical advice/ attention.
P302+P352	IF ON SKIN: Wash with plenty of soap and water.
P501	Dispose of contents/ container in accordance with local/ regional/ national/ international regulations.

Substrate Solution (S)
The TMB-Substrate (S) contains 3,3´,5,5´ Tetramethylbencidine (<0.05%)

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.

Stopping Solution (SL)
The Stopping solution contains 0.2 M acid sulphur acid (H_2SO_4)

H290	May be corrosive to metals.
H314	Causes severe skin burns and eye damage.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P301+P330+	IF SWALLOWED: rinse mouth.
P331	Do NOT induce vomiting.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.
P309+P310	IF exposed or if you feel unwell: Immediately call a POISON CENTER or doctor/physician.

4.1 General first aid procedures:

Skin contact: Wash affected area rinse immediately with plenty of water at least 15 minutes. Remove 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: After swallowing the product, if the affected person is conscious, rinse out the mouth with plenty of water: seek medical advice immediately.

5 SAMPLES

5.1 Sample type

Serum and Plasma:
Beside serum also EDTA- and Heparin- plasma samples can be used because in five corresponding serum/plasma samples no difference between leptin concentrations of >30% was detected. Citrate plasma was also tested in two samples. The measured leptin concentration was 93 and 81% of the serum concentration. Thus, none of the tested anticoagulants interfered with the Leptin measurement.

5.2 Specimen collection

The blood sample for serum preparation should be gained according to standardized venipucture procedure. Hemolytic reactions have to be avoided.
Leptin levels show a circadian variation with a peak during the night at about 2 a.m. (37). This variation together with the influence of food intake needs to be taken into account, when blood samples are collected.
Required sample volume: 20 µL for single / 40 µL for duplicate measurement

5.3 Sample stability

In firmly closable sample vials

- Storage at 20-25°C: 2 days
- Storage at -20° C: min. 2 years
- Freeze-thaw cycles max. 5

The storage of samples over a period of 2 years at -20°C, showed no influence on the reading. Freezing and thawing of samples should be minimized, 5 Freezing-Thawing showed no effect on samples.

5.4 Interference

Hemoglobin, Triglyceride and bilirubin in the sample do not interfere to a concentration of **1 mg/mL**, **100 mg/mL** and **100 µg/mL.** However, the use of hemolytic, lipemic or icteric samples should be validated by the user.

5.5 Sample dilution

- There is no special sample treatment necessary. The samples can be used undiluted (20 µL).

6 MATERIALS

6.1 Materials provided

The reagents listed below are sufficient for 96 wells including the standard curve.

SORB MT	**Microtiter plate, MTP** ready for use, coated with mouse-anti-Leptin-antibody. Wells are separately breakable.	**(8x12) wells**
CAL	**Standards A-E**, lyophilized, (recombinant human Leptin), concentrations are given on vial labels and on quality certificate in ng/mL.	**5 x 750 µL**
CONTROL 1	**Control Serum 1, KS1** lyophilised, (human serum), concentration is given on quality certificate in ng/mL.	**1 x 500 µL**
CONTROL 2	**Control Serum 2, KS2** lyophilised, (human serum), concentration is given on quality certificate in ng/mL.	**1 x 500 µL**
ENZ CONJ	**Antibody-HRP-Conjugate, AK** ready for use, mouse-anti-hLeptin-antibody biotinylated + streptavidin horseradish peroxidase conjugate	**1 x 12 mL**
DIL	**Dilution Buffer, VP** ready for use	**1 x 25 mL**
WASH SOLN 20x	**Washing Buffer, WP** 20-fold concentrated solution	**1 x 50 mL**
SUB TMB	**Substrate, S** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised tetramethylbencidine.	**1 x 12 mL**
STOP SOLN	**Stopping Solution, SL** ready for use, 0.2 M sulphuric acid.	**1 x 12 mL**
-	**Sealing Tape,** for covering the **microtiter plate.**	**2 x**
	**Instructions for use**	**1 x**
--	**Quality Certificate**	**1 x**

6.2 Materials required, but not provided

- Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer **WP (A. dest.),** 950 mL.
- Precision pipettes and multichannel pipettes with disposable plastic tips
- Vortex-mixer
- Microtiter plate shaker (350 rpm)
- Microtiter plate washer (recommended)
- Micro plate reader ("ELISA-Reader") with filter for 450 and ≥590 nm

7 TECHNICAL NOTES

Storage Conditions

Store the kit at 2-8°C after receipt until its expiry date. The lyophilized reagents should be stored at –20 °C after reconstitution. Avoid repeated thawing and freezing.

Storage Life

The shelf life of the components **after initial opening** is warranted for **4 weeks**, store the unused strips and microtiter wells **airtight** together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The **reconstituted components** standards **A-E** and Control Sera **KS1 and KS2** must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Up to 3 of the freeze-thaw cycles did not influence the assay. The 1:20 diluted Washing Buffer **WP** is 4 weeks stable at 2-8°C

Preparation of reagents

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. Reagents with different lot numbers cannot be mixed.

Reconstitution

The Standards **A – E** and Control Sera **KS1** and **KS2** are reconstituted with the Dilution Buffer **VP**. 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.

Dilution

The required volume of Washing Buffer **WP** is prepared by 1:20 dilution of the provided 20fold concentrate with Aqua dest.

Assay Procedure

When performing the assay, Blank, Standards **A-E**, Controls **KS1, KS2** and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody Conjugate **AK** as well as the succeeding Substrate Solution **S** should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution **SL** should be added to the plate in the same order as Substrate Solution **S**.
All determinations (Blank, Standards **A-E**, Controls **KS1**, **KS2** and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

Incubation

Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate Solution **S**, stabilised Tetramethylbencidine, is photosensitive–store and incubation in the dark.

Shaking

The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. We are recommending **200 to max.350 rpm**. Due to certain technical differences deviations may occur, in case the rotation frequency must be 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.

Washing

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 **WP** diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least.

The danger of handling with potentially infectious material must be taken into account.

When using an **automatic microtiter** plate washer, the respective instructions 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 dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

8 ASSAY PROCEDURE

Preparation of reagents		**Reconstitution:**	**Dilution**
A-E	**Standards**	in **750 µL** Dilution Buffer **VP**	-
KS1 and KS2	**Control Sera**	in **500 µL** Dilution Buffer **VP**	-
WP	**Washing Buffer**	-	**1:20** with **Aqua dest.**
Sample dilution is in general not necessary, use **20 µl undiluted** per determination.			
Before assay procedure bring all reagents to room temperature **20-25°C**.			

Assay Procedure in Double Determination:

Pipette	**Reagents**	**Position**
100 µL	Dilution Buffer **VP**	**Pipette in all required wells**
20 µL	Dilution Buffer **VP** (**Blank**)	A1/A2
20 µL	Standard **A** (**1 ng/mL**)	B1/B2
20 µL	Standard **B** (**10 ng/mL**)	C1/C2
20 µL	Standard **C** (**25 ng/mL**)	D1/D2
20 µL	Standard **D** (**50 ng/mL**)	E1/E2
20 µL	Standard **E** (**100 ng/mL**)	F1/F2
20 µL	Control Serum **KS1** (undiluted)	G1/G2
20 µL	Control Serum **KS2** (undiluted)	H1/H2
20 µL	Sample (undiluted)	in the rest of the wells according the requirements
Cover the wells with the sealing tape.		
Sample Incubation: 1 h at 20-25°C, 200 - 350 rpm		
5 x 300 µL	Aspirate the contents of the wells and **wash** 5 x with 300 µL each Washing Buffer **WP**/ **well**	In each well
100 µL	Antibody-POD-Conjugate **AK**	In each well
Cover the wells with the sealing tape.		
Incubation: 30 Minutes at 20-25°C, 200 - 350 rpm		
5 x 300 µL	Aspirate the contents of the wells and **wash** 5 x with 300 µL each Washing Buffer **WP**/ **well**	In each well
100 µL	Substrate Solution **S**	In each well
Incubation: 15 Minutes in the Dark at 20-25°C		
100 µL	Stopping Solution **SL**	In each well
	Measure the absorbance within 30 min at **450 nm** with ≥ 590 nm as reference wavelength.	

9 QUALITY CONTROL

Good laboratory practice requires that controls are included in each assay. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards/laws. All standards and kit controls must be found within the acceptable ranges as stated on the QC Certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

9.1 Quality criteria

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.25, and the absorbance of standard E should be above 1.00.
Samples, which yield higher absorbance values than Standard E, should be re-tested with a dilution.

10 EVALUATION OF RESULTS

10.1 Establishing of the standard curve

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

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 samples and standards

3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.

4) Recommendation: Calculation of the standard curve should be done by using a computer program, because the curve is in general (without respective transformation) not ideally described by linear regression. **A higher-grade polynomial, or four parametric logistic (4-PL) curve fit** or **non-linear regression** are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).

5) The Leptin concentration in ng/mL of the samples and controls **KS1** and **KS2** is calculated automatically by your program.

10.2 Example of a typical standard curve

The following data is for demonstration only and cannot be used in place of data generation at the time of assay.

	Blank	A	B	C	D	E
ng/mL	0.0	1	10	25	50	100
$OD_{(450\text{-}620\ nm)}$	0.04	0.083	0.680	1.449	2.165	2.764

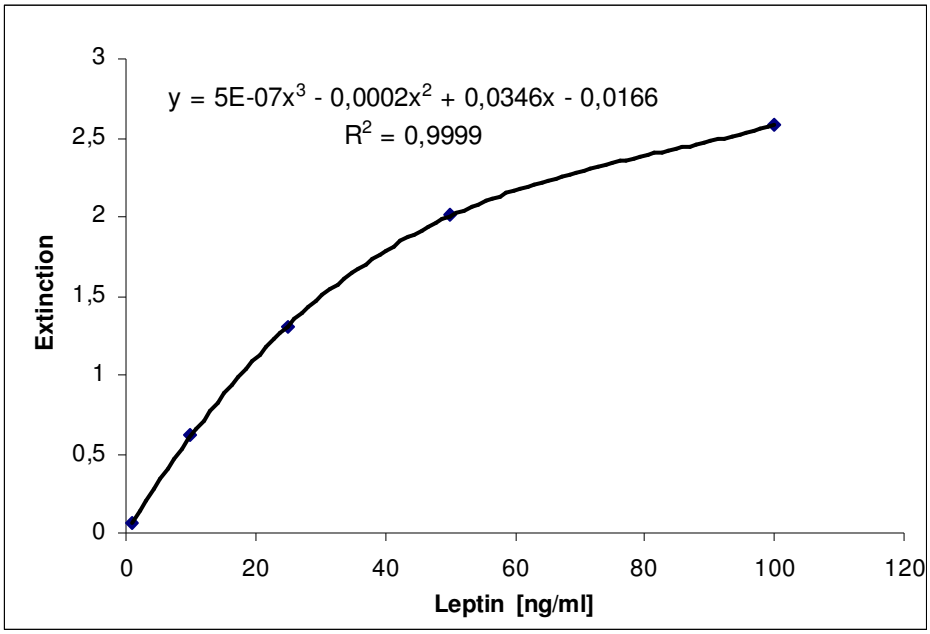

Figure 1 Examplary standard curve

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

10.3 Exemplary calculation of Leptin concentrations

Measured extinction of your sample 0.39
Measured extinction of the blank 0.04

Your measurement programm will calculate the Leptin concentration of the diluted sample automatically by using the difference of sample and blank for the calculation. You only have to determine the most suitable curve fit (here: polynomial 3rd degree).

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

$$0.35 = 5 \times 10^{-7} x^3 - 0.0002x^2 + 0.0346x - 0.0166$$
$$7.012 = x$$

as the sample is undiluted the Leptin concentration of the sample is

7.012 ng/mL

10.4 Interpretation of results

The test results should not be the only base for therapeutic decisions. The results should be interpreted in regard to anamnesis, further clinical observations and results of other diagnostic investigations. Further, it is recommended to establish reference and cut-off values corresponding to the relevant group of patients for each laboratory.

10.5 Limitation of procedure

The Demeditec sensitive human Leptin ELISA, DEE007 is based on monoclonal antibodies. Generally, this technique could be sensible to heterophilic antibodies or rheumatic factors in the sample. Their influence is reduced by assay design, but cannot be excluded completely.

11 REFERENCE 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 2-9 and tables 1 - 9). 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 1 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:

$$Leptin = 0.0181 \cdot e^{(0.2067 \cdot BMI)}$$

The 5th percentile is given by: $$Leptin = 0.0181\ e^{(0.2067 \cdot BMI - 1.1919)}$$

and the 95th percentile is given by: $$Leptin = 0.0181\ e^{(0.2067 \cdot BMI + 1.1919)}$$

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:

$$\text{leptin SDS} = (\ln(\text{leptin}) - \ln(a) - b \cdot \text{BMI}) / d$$

In this equation, ln represents the natural logarithm (referring to the basis e). The constants a, b and d are given in table 1 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

Table 1 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 (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 2 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	121.0			
36	113.0				
37	145.0				

Table 3 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				

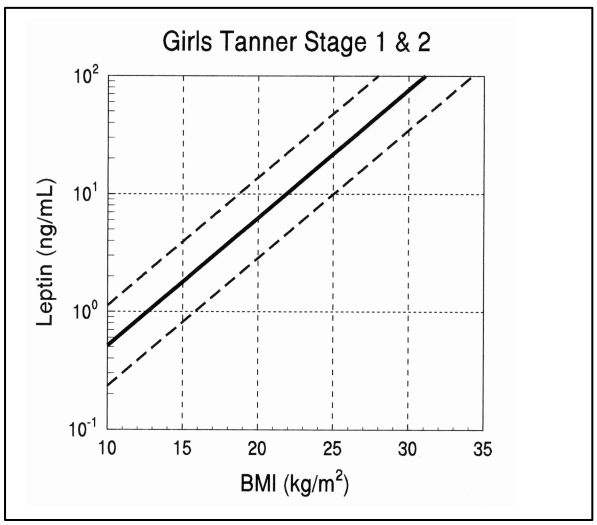

Figure 2 Reference ranges of human serum levels referring to BMI: Girls Tanner stage 1 & 2 (see text for details)

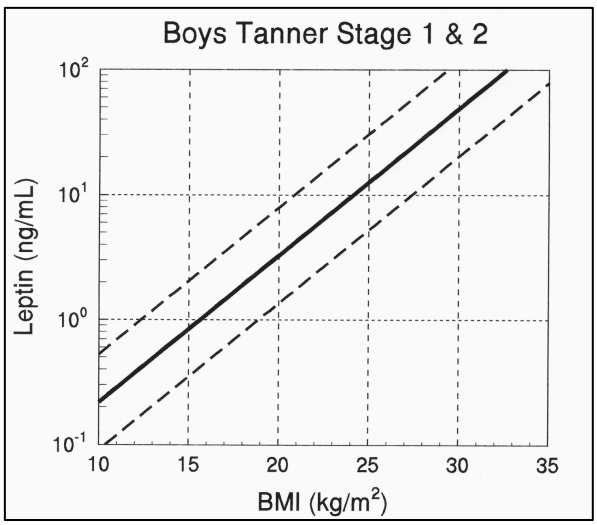

Figure 3 Reference ranges of human serum levels referring to BMI: Boys Tanner stage 1 & 2 (see text for details)

Table 4 Girls Tanner stages 3 and 4

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				

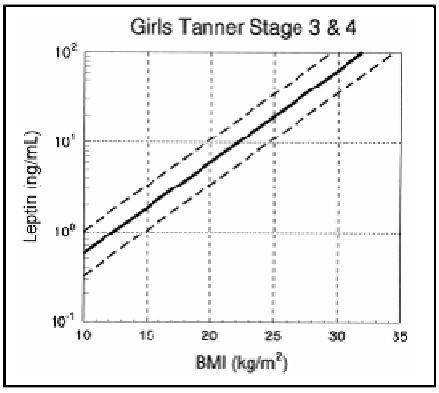

Figure 4 Reference ranges of human serum levels referring to BMI: Girls Tanner stage 3 & 4 (see text for details).

Table 5 Boys Tanner stages 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	37.9	124.0	
38	8.7	14.2	46.7		
39	10.7	17.4	57.4		
40	13.1	21.4	70.5		

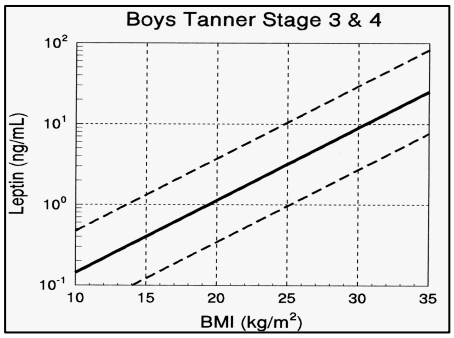

Figure 5 Reference ranges of human serum levels referring to BMI: Boys Tanner stage 3 & 4 (see text for details).

Table 6 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		

Table 7 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

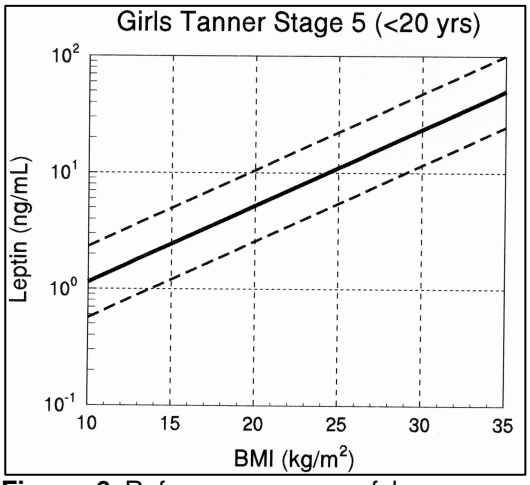

Figure 6 Reference ranges of human serum levels referring to BMI: Girls Tanner stage 5 (see text for details)

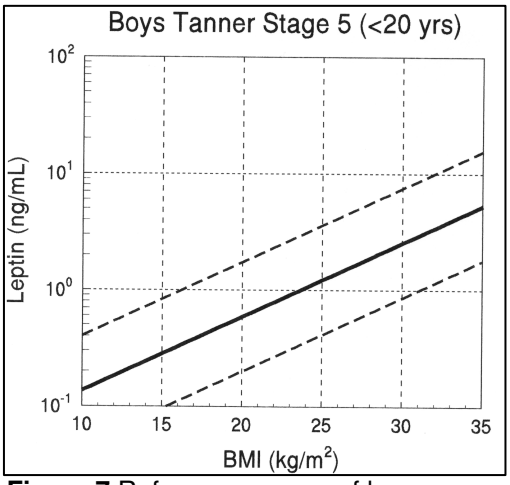

Figure 7 Reference ranges of human serum levels referring to BMI: Boys Tanner stage 5 (see text for details).

Table 8 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		

Table 9 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		

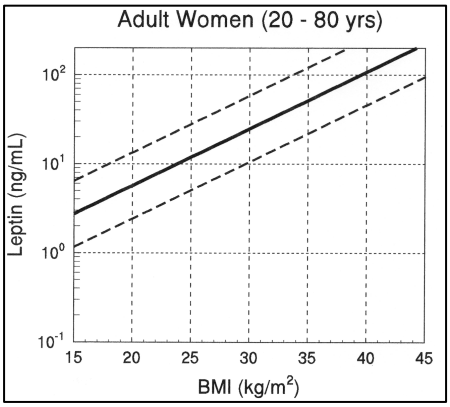

Figure 8 Reference ranges of human serum levels referring to BMI: Adult women (see text for details)

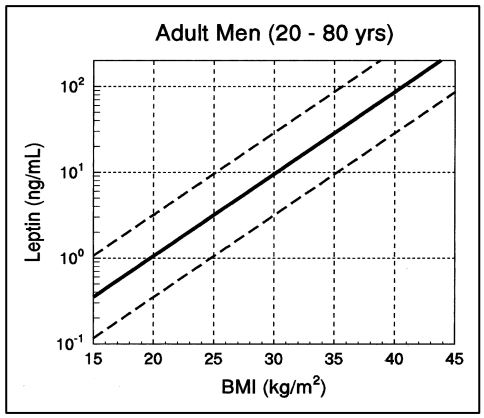

Figure 9 Reference ranges of human serum levels referring to BMI: Adult men (see text for details).

12 PERFORMANCE CHARACTERISTICS

12.1 Sensitivity

Limit of Detection / analytical sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the blank + 2SD. The analytical sensitivity of the DEE007 is <0.25 ng/ml (different determinations with range of 0.012 up to 0.24 ng/mL; with a mean of 0.095 ng/mL).
Based on the undiluted sample the limit of quantification is 1 ng/mL.

12.2 Precision

Intra-Assay Variance & Accuracy
Two samples were measured eight times in three different assays. Exemplary results are shown in Table 10, here the deviation of the target value is 6.3 and 7%. The measured coefficient of variation (CV) is 4.26% on average (n=6).

Table 10: Intra-Assay Variation

	Target value [ng/mL]	[n]	Mean [ng/mL]	Standard Deviation [ng/mL]	CV [%]
Sample 1	85	8	90.36	4.68	5.18
Sample 2	6	8	6.42	0.3	4.71

Inter-Assay Variance & Accuracy
Precision was evaluated by measuring the leptin content of the same serum samples several times in independent assays. On average the coefficient of variation was 12% (SD 3.5) and the deviation of the target value was less than 20% in 90% of the tested samples. The results are summarized in table 11.

Table 11 Inter-Assay variability and accuracy. Results are based on 835 measurement within 10 years conducted with 15 different lots.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
Mean [ng/mL]	11.11	5.64	37.06	2.38	6.18	7.21	4.65	4.35	2.45	36.08	5.93	25.12	17.54
SD [ng/mL]	0.92	0.57	3.26	0.43	0.64	0.57	0.49	0.61	0.47	3.68	0.75	2.63	1.66
Number [n]	121	99	28	182	86	86	79	23	27	25	22	26	31
CV [%]	8.33	10.10	8.81	17.97	10.35	7.88	10.57	14.04	19.21	10.19	12.57	10.45	9.46

12.3 Linearity

Samples are routinely used undiluted. Dilution of 1:2 up to 1:20 have been tested with two serum samples and the recalculated amount of measured Leptin was not significant different of the amount in the undiluted sample. This however seems to be dependent on the sample. Two other samples were diluted up to 1:80 and showed insufficient linearity (Table 12), thus a dilution higher than 1:10 is not recommended.

Table 12 Linearity of sample dilution. The recalculated leptin concentrations of differently diluted human serum samples are shown here. The dilutions were treated like samples and the Demeditec Leptin ELISA DEE007 was conducted as described in the package insert.

Dilution	Sample 1 [µg/L]	Sample 2 [µg/L]	Dilution	Sample 3 [µg/L]	Sample 4 [µg/L]
undiluted	35.2	17.8	undiluted	36.8	10.5
1:2	35.0	17.3	1:2	41.3	12.7
1:5	38.6	18.0	1:4	41.4	14.1
1:10	35.5	17.7	1:10	39.9	15.7
1:20	40.6	15.9	1:30	54.5	18.6
-	-	-	1:60	57.1	26.1
-	-	-	1:80	55.6	28.5
Mean [µg/L]	36.96	17.4	-	46.67	18.01
Standard Deviation [µg/L]	2.5	0.86	-	8.65	6.86
Coefficient of Variation [%]	6.8	4.97	-	18.54	38.06

12.4 Recovery

Recombinant leptin in Dilution Buffer (VP) was used to enrich human serum samples. The leptin content of the so enriched samples was measured and recovery in comparison to the theoretical Leptin amount was calculated. Results are shown in table 13 and demonstrate that the two exemplary samples do not contain substances interfering with leptin measurement.

Table 13 Recovery of recombinant human Leptin in Buffer & Serum samples

		recombinant Leptin		Recovery [%]	
	w/o Leptin	5 µg/L	10 µg/L	5 µg/L	10µg/L
Buffer	-	5.79	11.78	116	118
Sample 1	5.74	10.72	15.36	100	98
Sample 2	4.38	9.67	14.63	103	102

The results shown in Table 13 demonstrate that the two tested samples did not contain substances interfering with Leptin measurement. Further, the traceability of the test system to the international standard WHO/NIBSC 97/594 was evaluated. Therefore the NIBSC standard was diluted to 10, 20 and 40 ng/mL and used as sample. Recovery based on the nominal Leptin content of the NIBSC Standards was 106, 113 and 102% respectively.

12.5 Interference

Interference of bilirubin, haemoglobin and triglycerides was tested by adding different amounts of these substances to human serum containing Leptin. For comparison the same amount of buffer without any substance was also added to the serum. Table 14 demonstrates that neither bilirubin nor triglycerides or haemoglobin exert any influence on the measurement of Leptin in human serum.

Table 14 Interference of physiological substances

	Triglyceride	Bilirubin	Hemoglobin
	100 mg/mL	**100 µg/mL**	**1 mg/mL**
Serum 1	95	101	94
Serum 2	107	105	105
Serum 3	111	101	101

13 COMPARISON STUDIES

Here the Demeditec Assay is compared with three different commercially available test systems demonstrating a good correlation between both assays (Figure 10).

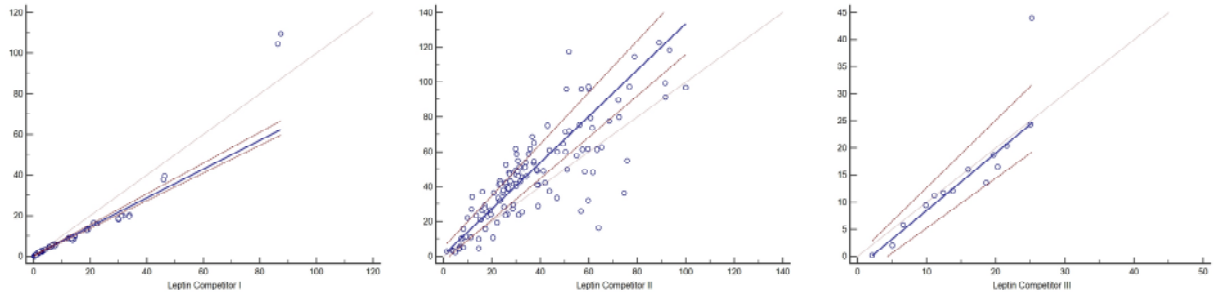

Figure 10 Assay Comparison. Leptin [ng/mL] of different sample panels (n=80/127/14) was evaluated by three different commercially available immunoassays and Demeditec DEE007. Results were analysed by Passing-Bablok regression. A significant deviation from linearity (Cusum test) is seen with competitor I. The analysis revealed the following linear equations and residual standard deviations:

Competitor I y = 0.359 + 0.709x (6.013)
Competitor II y = 0.713 + 1.329x (10.372)
Competitor III y = -2.067 + 1.057x (4.0173)

Instructions for use for scientific application

14 SCIENTIFIC APPLICATION

In addition to serum and plasma samples Leptin can be determined in other human body fluids and in cell culture supernatants of human cell lines for research purposes.

14.1 Samples suitable for scientific application

Serum, plasma, saliva, urine and cell culture supernatant of human cell lines
Serum and plasma samples are recommended to use undiluted.
In the other samples, the Leptin levels can vary considerable, the optimal dilution must be found out by the customer.

Table 15 Results of sample matrix tests. Leptin was added to the respectively diluted samples. Enriched samples were measured without further dilution. Shown is the relative recovery of added leptin of the value measured in enriched assay buffer.

Dilution	Sample	[ng/mL]	Recovery [%]
-	Dilution Buffer (VP) + recombinant Leptin	4.342	--
-	Saliva	0.247	--
1:2	Saliva + rec. Leptin	4.738	103
1:5	Saliva + rec. Leptin	4.354	95
1:10	Saliva + rec. Leptin	4.408	96
1:20	Saliva + rec. Leptin	4.343	95
-	Urine	0.203	--
1:2	Urine + rec. Leptin	4.486	99
1:5	Urine + rec. Leptin	5.101	112
1:10	Urine + rec. Leptin	5.316	117
1:20	Urine + rec. Leptin	4.492	99
-	Cell culture medium	0.003	--
1:2	Cell culture medium + rec. Leptin	3.922	90
1:5	Cell culture medium + rec. Leptin	5.571	128
1:10	Cell culture medium + rec. Leptin	5.112	118
1:20	Cell culture medium + rec. Leptin	3.63	84
without added Leptin			
Dilution	**Sample**	**[ng/mL]**	**Recovery [%]**
1:1	Amniotic fluid	21.981	100
1:2	Amniotic fluid	21.812	99
1:4	Amniotic fluid	19.236	88
1:8	Amniotic fluid	18.537	84
1:16	Amniotic fluid	17.876	81

14.2 Species Cross-Reactivity

Serum of the cited species was diluted and used as sample in this assay system.

No signal was detected in serum of the following species:
Horse, Cow, Chicken, Rabbit, Dog, Guinea pig, Sheep, Mouse, Goat, Donkey, Rat, Cat, Pig.

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17 RIASSUNTO DELLA PROCEDURA Leptin ELISA DEE007

Ricostituzione/diluizione di reagenti e campioni		
Standards A-E	Diluition Buffer VP dopo l'uso conservare a -20°C	**750 µL**
Control Sera KS1 & KS2	Diluition Buffer VP dopo l'uso conservare a -20°C	**500 µL**
Washing Buffer WP	(es. aggiungere l'intero contenuto della bottiglia (50 mL) in un contenitore graduato e portare a volume di 1000 mL)	**1:20**
La diluizione dei campioni non è necessaria, utilizzare 20 µL di campione non diluito		
Prima del dosaggio portare tutti i reattivi a temperatura ambiente.		

Procedura in doppia determinazione

Quantità	**Reattivi**	**Posizione pozzetto**
100 µL	Dilution Buffer **VP**	**Pipettare in tutti I pozzetti necessari**
20 µL	Dilution Buffer **VP**	A1/2
20 µL	Standard **A (1 ng/mL)**	B1/2
20 µL	Standard **B (10 ng/mL)**	C1/2
20 µL	Standard **C (25 ng/mL)**	D1/2
20 µL	Standard **D (50 ng/mL)**	E1/2
20 µL	Standard **E (100 ng/mL)**	F1/2
20 µL	Control Serum **KS1**	G1/2
20 µL	Control Serum **KS2**	H1/2
20 µL	campione nei	Pozzetti successivi
Coprire i pozzetti con il foglio adesivo		
Incubazione: 1 h a RT, (20-25°C), **200 - 350 rpm**		
5 x 300 µL	Aspirare il contenuto dei pozzetti e **lavare** 5x con 300 µL di Wash Buffer **WP**	Tutti i pozzetti
100 µL	Antibody (**AK**)-POD-Conjugate	Tutti i pozzetti
Incubazione: 30 min a RT, (20-25°C), **200 - 350 rpm**		
5 x 300 µL	Aspirare il contenuto dei pozzetti e **lavare** 5x con 300 µL di Wash Buffer **WP**	Tutti i pozzetti
100 µL	Substrate Solution **S**	Tutti i pozzetti
Incubazione: 15 min. al **buio** a RT (20-25°C),		
100 µL	Stop Solution **SL**	Tutti i pozzetti
Misurare l'assorbanza entro 30 min. a **450 nm** (Reference ≥ 590 nm)		

18 Internationale Assay Description

A-E	**CAL**	**Rec in 750 µL DIL VP**	
KS1	**Control**	**Rec in 500 µL DIL VP**	
KS2	**Control**	**Rec in 500 µL DIL VP**	
WP	**WASH SOLN 20x**	**-**	**1:20 DILU A. dest.**
-	**SPE Sample**		
-	**°C 20-25 °C**		

100 µL	**DIL VP**	**A1 - End**
20 µL	**DIL VP**	A1/A2
20 µl	**CAL A (1 ng/mL)**	B1/B2
20 µl	**CAL B (10 ng/mL)**	C1/C2
20 µl	**CAL C (25 ng/mL)**	D1/D2
20 µl	**CAL D (50 ng/mL)**	E1/E2
20 µl	**CAL E (100 ng/mL)**	F1/F2
20 µl	**CONTROL 1 KS1**	G1/G2
20 µl	**CONTROL 2 KS2**	H1/H2
20 µl	**SPE Sample**	
	TAPE	
	1 h °C 20-25 ↔ 200 - 350 rpm	
5x 300 µL	**5x WASH SOLN WP**	
100 µL	**ENZ CONJ AK**	
	TAPE	
	0.5 h °C 20-25 ↔ 200 - 350 rpm	
5x 300 µL	**5x WASH SOLN WP**	
100 µL	**SUB TMB S**	
	0.25 h °C 20-25	
	STOP SOLN SL	
	MEASURE	

SYMBOLS USED WITH DEMEDITEC ASSAYS

Symbol	English	Deutsch	Francais	Espanol	Italiano
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conforme aux normes européennes	Conformidad europea	Conformità europea
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las Instrucciones	Consultare le istruzioni per l'uso
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Ussage Diagnostic in vitro	Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Référence	Número de catálogo	No. di Cat.
LOT	Lot. No. / Batch code	Chargen-Nr.	No. de lot	Número de lote	Lotto no
Σ	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Note warnings and precautions	Warnhinweise und Vorsichtsmaßnahmen beachten	Avertissements et mesures de précaution font attention	Tiene en cuenta advertencias y precauciones	Annoti avvisi e le precauzioni
	Storage Temperature	Lagerungstemperatur	Temperature de conservation	Temperatura de conservacion	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributtore

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