

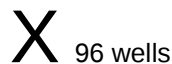
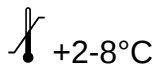
# Leptin ELISA

Enzyme Immunoassay for Quantitative Determination of

**human Leptin**

English

**For Research Use Only.  
Not for use in diagnostic procedures.**



**h E07**



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according to DIN EN ISO 15223-1

|                                                                                     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|    | Expiry date/ Verfallsdatum/ Date de péremption/ Data di scadenza/ Fecha de caducidad/ Data de validade/ Uiterste gebruiksdatum/ Udløbsdato/ Bäst före-datum/ Termin ważności/ Lejárati idő/ Čas expirácie/ Doba expirace/ Срок на годност/ Aegumiskuupäev/ Ημερομηνία λήξης/ Data de expirare/ Rok uporabe/ Viimeinen käyttöpäivä                                                                                                                                                                                                                                                                                                                                                                                                         |
|    | Consider instructions for use/ Bitte Gebrauchsanweisung beachten/ Consultez la notice d'utilisation/ Consultare le istruzioni per l'uso/ Consulte las instrucciones de uso/ Respeitar as instruções de utilização/ A.u.b de gebruiksaanwijzing volgen/ Se brugsanvisningen/ Läs anvisningarna före användning/ Proszę przeczytać instrukcję obsługi/ Vegye figyelembe a használati utasításban foglaltakat/ Postupujte podľa pokynov na použitie/ Dodržujte návod k použití/ Моля, спазвайте инструкцията за употреба/ Palun järgige kasutusjuhendit/ Λάβετε υπόψη σας τις οδηγίες χρήσης/ Vă rugăm să respectați instrucțiunile de utilizare/ Upošteвайте navodila za uporabo/ Lue käyttöohje huolellisesti!                             |
|    | Lot-Batch Number/ Charge-Chargennummer/ Lot-Code du lot/ Lotto-Numero di lotto/ Lote-Código de lote/ Lote-Código do Lote/Lot-Partijnummer/ Lot-Batchkode/ Partisatskod/ Numer serii/ Tétel-sarzs szám/ Číslo šarže/ Číslo šarže/ Партиден номер/Partii number/ Παρτίδα-αριθμός παρτίδας/ Lot-număr lot/ Številka serije/ Erä                                                                                                                                                                                                                                                                                                                                                                                                              |
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|    | Catalogue Number/ Bestellnummer/ Numéro de référence/ Numero di riferimento/ Número de referencia/ Número de Referência/ Referentienummer/ Referencenumber/ Beställningsnummer/ Numer katalogowy/ Rendelési szám/ Katalógové číslo/ Objednací číslo/ Каталоген номер/ Tellimisnumber/ Αρ. παραγγελίας/Număr comandă/ Številka naročila/ Viite tai tilausnumero                                                                                                                                                                                                                                                                                                                                                                            |
|    | Store at between/ Lagerung bei zwischen/ Conserver à entre/ Conservare a tra/ Conservar a temp. entre/ Armazenar entre/ Bewaar bij tussen/ Opbevares mellem/ Förvaras vid/ Przechowywać w/ Tárolási tartomány/ Skladujte v rozsahu / Skladujte v rozmezí/ Температурно ограничение/ Säilitada temperatuuridel/ Φύλαξη σε θερμοκρασία/ Depozitare între/ Skladičenje med/ Sailytys x-y Celsiusasteen lämpötilassa                                                                                                                                                                                                                                                                                                                          |
|  | Contains sufficient for x tests/ Inhalt ausreichend für x Tests/ Contient suffisant pour x tests/ Contenuto sufficiente per x test/ Contenido suficiente para x pruebas/ Conteúdo suficiente para x testes/ Bevat voldoende voor x bepalingen/ Indeholder tilstrækkeligt til x prøver/ Innehållet räcker till x analyser/ Zawartość na x testów/ Tartalma x teszt elvégzésére elegendő/ Obsahuje materiál pre x testov/ Obsah dostačuje pro x testů/ Съдържание достатъчно за x тестове/ Sisust jätkub x katse jaoks/ Το περιεχόμενο επαρκεί για x δοκιμές/ Conținut suficient pentru x teste/ Vsebina zadostuje za x preizkusov/ Sisältö riittää x testille                                                                              |
|  | Incubation time/ Inkubationszeit/ Temps d'incubation/ Tempo d'incubazione/ Tiempo de incubación/ Tempo de incubação/ incubatietijd/ Inkubationstid/ inkubationstid/ Czas inkubacji/ Inkubációs idő/ Inkubačná lehota/ Inkubační doba/ Инкубационен период/ Inkubatsiooniaeg/ Χρόνος επώασης/ Timp de incubare/ Inkubacijska doba/ inkubaatioaika                                                                                                                                                                                                                                                                                                                                                                                          |
|  | Incubate at/ Inkubation bei/ Incuber à/ Incubare a/ incubar a/ Incubar a/ incubatietemperatuur/ Inkubation ved/ inkubation vid/ Inkubacja przy/ Inkubáció hőmérséklete/ Inkubácia pri/ Inkubace při/ Инкубира се при/ Inkubatsioon temperatuuril/ Επώαση στους/ Incubare la/ Inkubacija pri/ Inkubaatiolämpötila                                                                                                                                                                                                                                                                                                                                                                                                                          |
|  | Mix tubes with a Vortex mixer/ Mix Röhrchen mit Vortex Mixer/ Mélanger à l'aide d'un vortex/ Miscelare la provetta con agitatore Vortex/ Tubos de mezcla con mezclador de vortex/ Misturar os tubos com um agitador Vortex/ buisjes mengen met een Vortex/ Blanderør med Vortex-mixer/ Blanda rören med en vortexblandare/ Miksowanie rurek w mikserze Vortex/ Csővecskék keverése örvénykeverővel/ Premiešat pomocou prístroja Vortex/ Promíchat pomocí přístroje Vortex/ Разбъркване на епруветките с миксер Vortex/ Segada torukesi Vortexi mikseriga/ Αναμίξτε τους σωληνίσκους με αναδευτήρα Vortex/ Amestecați eprubetele cu ajutorul unui agitator vortex/ Mešanje cevčic z mešalnikom Vortex/ Sekoita putket Vortex sekoittajalla |
| <b>MTP</b>                                                                          | Mikrotiterplate/ Mikrotiterplatte/ plaque de microtitrage/ Piastra di microtitolazione/ Placa de microtitulación/ Placa de Microtitulação/ Microtiterplaat/ Mikrotiterplade/ mikrotiterplatta/ microtiterplaat/ Płytką microtiter/ Mikrotiter lap/ Mikrotitračná podložka/ Mikrotitrační podložka/ Микротитърна плака/ Mikrotiterplaat/ Τρυβλίο μικροτιτλοδότησης/ Microplacă/ Mikrotitrská plošča/ Mikrotitruslevy                                                                                                                                                                                                                                                                                                                       |
|  | Reconstitute in/ Rekonstituieren in/ Reconstituer dans/ Ricostituire nel/ Reconstituir en/ Reconstituir em/ Reconstituieren in/ Rekonstituér i/ Rekonstituera/ Rekonstituować w/ Helyreállítás/ Znovu připravit za/ Znovu připravit za/ Разтваряне в/ Moodustada uuesti/ Ανασυστήστε σε/ Reconstituire în/ Predelava v/ Rekonstitui                                                                                                                                                                                                                                                                                                                                                                                                       |
|  | Sample/ Probe/ Echantillon/ Campione/ Muestra/ Amostra/ Monster/ Prøve/ prov/ Próbka/ Minta/ Vzorka/ Vzorek/ Проба/ Proov/ Δείγμα/ Probă/ Vzorec/ Näyte                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| <b>DET</b>                                                                          | Antibody and Enzyme Conjugate/ Antikörper- und Enzymkonjugat/ anticorps conjugué et conjugué enzymatique/ Coniugato di anticorpo ed enzima/ Conjugado de anticuerpos y enzimas/ Conjugado Anticorpo-Enzima/ antilichaam- en enzymconjugaat/ Antistoffer og enzym-konjugat/ antikropps- och enzymkonjugat (antikropp och enzym, konjugat)/ Konjugat antyciał i enzymów/ Antitest és enzim páros/ Protílatkový a enzymatický konjugát/ Protílatkový a enzymatický konjugát/ Антицяло и ензим конюгат/ Antikehad ja ensüümi konjugaat/ Σύμπλοκο αντι σώματος-ενζύμου/ Compuși din anticorpi și enzime/ Antitelesa in konjugat encima/ Vasta-aine ja entsymi konjugaatti                                                                      |
| <b>DIL</b>                                                                          | Dilution Buffer/ Verdünnungspuffer/ Tampon de dilution/ Tampone di diluizione/ Tampón de dilución/ / Tampão de diluição/ Verdunningsbuffer/ / Fortyndingsbuffer/ Utspädningsbuffert/ Bufor rozcieńczający/ / Hígító puffer/ Riediaci pufor/ Ředící pufir/ Буфер за разреждане/ Lahjenduspuhver/ Ρυθμιστικό διάλυμα αραιώσης/ / Tampon de diluare/ Pufer za redčenje/ Laimennuspuskuri                                                                                                                                                                                                                                                                                                                                                     |



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## **INSTRUCTIONS FOR USE**

| <b>Leptin ELISA E07</b>      | <b>96 Determinations</b>                                     |
|------------------------------|--------------------------------------------------------------|
| Principle of the test        | Enzyme-linked Immunoassay                                    |
| Duration (incubation period) | 1.75 h                                                       |
| Antibodies                   | monoclonal antibodies                                        |
| Buffer                       | Ready for use and 20fold concentrate                         |
| Calibrators                  | 5 single Calibrators: 1 – 100 µg/L, recombinant human Leptin |
| Reference material           | International Standard WHO/ NIBSC 97/594, recombinant Leptin |
| Assay Range                  | 0.25 – 100 µg/L                                              |
| Controls                     | 2 Controls, freeze-dried                                     |
| Sample                       | human serum / plasma                                         |
| Required sample volume       | 20 µL or 40 µL for single or duplicate measurement           |
| Sample dilution              | undiluted                                                    |
| Analytical sensitivity       | ø £ 0.25 µg/L                                                |
| Intra- / Interassay Variance | ø < 15%                                                      |

## 1 INTENDED USE

Measurement of human Leptin in human serum, plasma, urine and saliva for research use!

## 2 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 enzyme immunoassay kit is suited for measuring human leptin in serum or plasma, and conditioned adipocyte culture media.

### **3 ASSAY PRINCIPLE**

The Mediagnost ELISA for Leptin E07 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 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 closing substrate reaction, the turn of the colour will be catalysed quantitatively depending on the Leptin-level of the samples.

### **4 WARNINGS AND PRECAUTIONS**

For research and professional use only.

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. Mediagnost 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. Reagents with different lot numbers cannot be mixed.

Temperature WILL affect the absorbance readings of the assay. However, values for the patient samples will not be affected.

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 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.

Spilled material must be wiped off immediately and should become disinfected. Clean contaminated areas and equipment with a suitable detergent.

**Caution: This kit contains material of human and/or animal origin. Therefore, all components and 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: **Controls CTR1 and CTR2**

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 CAL A-E, DET, DIL, WB

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 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. |

#### Stop Solution STP

The Stop Solution contains 0.2 M acid sulphur acid (H<sub>2</sub>SO<sub>4</sub>)

|            |                                                                                         |
|------------|-----------------------------------------------------------------------------------------|
| 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 as well as cell culture medium, urine and saliva are applicable.

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 venipuncture 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.

### 5.3 Required sample volume:

20 µL for single / 40 µL for duplicate measurement

### 5.4 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.

### 5.5 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.

### 5.6 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 calibration curve.

|                                                                                     |                                                                                                                                            |                     |
|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| <b>MTP</b>                                                                          | <b>Microtiter plate</b> , ready for use, coated with mouse-anti-Leptin-antibody. Wells are separately                                      | <b>(8x12) wells</b> |
| <b>CAL A-E</b>                                                                      | <b>Calibrators</b> , lyophilized, (recombinant human Leptin), concentrations are given on vial labels and on quality certificate in ng/mL. | <b>5 x 750 µL</b>   |
| <b>CTR1</b>                                                                         | <b>Control 1</b> , lyophilised, (human serum), concentration is given on quality certificate in ng/mL.                                     | <b>1 x 500 µL</b>   |
| <b>CTR2</b>                                                                         | <b>Control 2</b> , lyophilised, (human serum), concentration is given on quality certificate in ng/mL.                                     | <b>1 x 500 µL</b>   |
| <b>DET</b>                                                                          | <b>Antibody-HRP-Conjugate</b> , ready for use, mouse-anti-hLeptin-antibody biotinylated + streptavidin horseradish peroxidase conjugate    | <b>1 x 12 mL</b>    |
| <b>DIL</b>                                                                          | <b>Dilution Buffer</b> , ready for use                                                                                                     | <b>1 x 25 mL</b>    |
| <b>WB</b>                                                                           | <b>Washing Buffer</b> , 20-fold concentrated solution                                                                                      | <b>1 x 50 mL</b>    |
| <b>S</b>                                                                            | <b>Substrate</b> , ready for use, horseradish-peroxidase-(HRP) substrate, stabilised tetramethylbenzidine.                                 | <b>1 x 12 mL</b>    |
| <b>STP</b>                                                                          | <b>Stop Solution</b> , ready for use, 0.2 M sulphuric acid.                                                                                | <b>1 x 12 mL</b>    |
| -                                                                                   | <b>Sealing Tape</b> , for covering the <b>microtiter plate</b> .                                                                           | <b>2 x</b>          |
|  | <b>Instructions for use</b>                                                                                                                | <b>1 x</b>          |
| -                                                                                   | <b>Quality Certificate</b>                                                                                                                 | <b>1 x</b>          |

### 6.2 Materials required, but not provided

- Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer **WB (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 <sup>3</sup> 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, protect from sunlight.

### Storage Life

The shelf life of the components **after initial opening** is warranted for **4 weeks, if stored appropriately**. 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**: Calibrators **A-E** and Controls **CTR1** and **CTR2** 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 **WB** 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 Calibrators **A – E** and Controls **CTR1** and **CTR2** are reconstituted with the Dilution Buffer **DIL**. 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 **WB** is prepared by 1:20 dilution of the provided 20fold concentrate with Aqua dest.

### Assay Procedure

When performing the assay, Blank, Calibrators **A-E**, Controls **CTR1**, **CTR2** and the samples should be pipette as fast as possible. To avoid distortions due to differences in incubation times, Antibody-HRP-Conjugate **DET** as well as the succeeding Substrate **S** should be added to the plate in the same order and in the same time interval as the samples. Stop Solution **STP** should be added to the plate in the same order as Substrate **S**.

All determinations (Blank, Calibrators **A-E**, Controls **CTR1**, **CTR2** 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 **S**, stabilised Tetramethylbencidine, is photosensitive—storage 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 **WB** 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 for 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

All determinations (Calibrators **A-E**, Controls **CTR1 & CTR2** 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, Controls and the samples should be pipette as fast as possible.

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

**Shaking intensity should not exceed 350 rpm!**

To avoid distortions due to differences in incubation times, Antibody-POD-Conjugate **DET** as well as the following Substrate **S** should be added to the plate in the same order and in the same time interval as the samples. Stop Solution **STP** should be added to the plate in the same order as the Substrate.

- 1) Pipette in all needed wells **100 µL** of Dilution Buffer **DIL**.
- 2) Add **20 µL** Dilution Buffer **DIL** to the first wells (blank). Subsequently, add **20 µL** of each Calibrator **CAL** or Control (**CTR1 & CTR2**) or **Sample** to the following wells.
- 3) Cover the wells with sealing tape and incubate the plate for **1 hour** shaking with **200 to max. 350 rpm**.
- 4) After incubation aspirate the contents of the wells into a disinfectant (possible theoretically risk of infection!) and wash the wells **5 times** with **300 µL** of Washing Buffer **WB** / well respectively. The Washing Buffer **WB** should incubate at least for 15 seconds/cycle
- 5) Pipette **100 µL** of the Antibody-POD-Conjugate **DET** in each well, cover the wells with sealing tape and incubate **30 minutes** shaking with **200 to max. 350 rpm**.
- 6) After incubation wash the wells **5 times** with Washing Buffer **WB** as described in step 4
- 7) Pipette **100 µL** of the Substrate **S** in each well.
- 8) Incubate the plate for **15 minutes in the dark at room temperature (20 - 25°C)**.
- 9) Stop the reaction by adding **100 µL** of Stop Solution **STP**.
- 10) Measure the colour reaction within 30 minutes at 450nm (reference **filter ≥590 nm**).

## 9 CALCULATION OF RESULTS

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 Calibrator E should be above 1.00.

Samples, which yield higher absorbance values than Calibrator E, should be re-tested with a dilution.

### 9.1 Establishing the calibration curve

| Calibrator | 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, controls and calibrators.
- 3) Plot the calibrator concentrations on the x-axis versus the mean value of the absorbance of the calibrators on the y-axis.
- 4) Recommendation: Calculation of the calibration 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 **CTR1** and **CTR2** is calculated automatically by your program.

### 9.2 Example of a typical Calibration 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(450-620 nm) | 0.04  | 0.083 | 0.680 | 1.449 | 2.165 | 2.764 |

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 0.39

Measured extinction of the blank 0.04

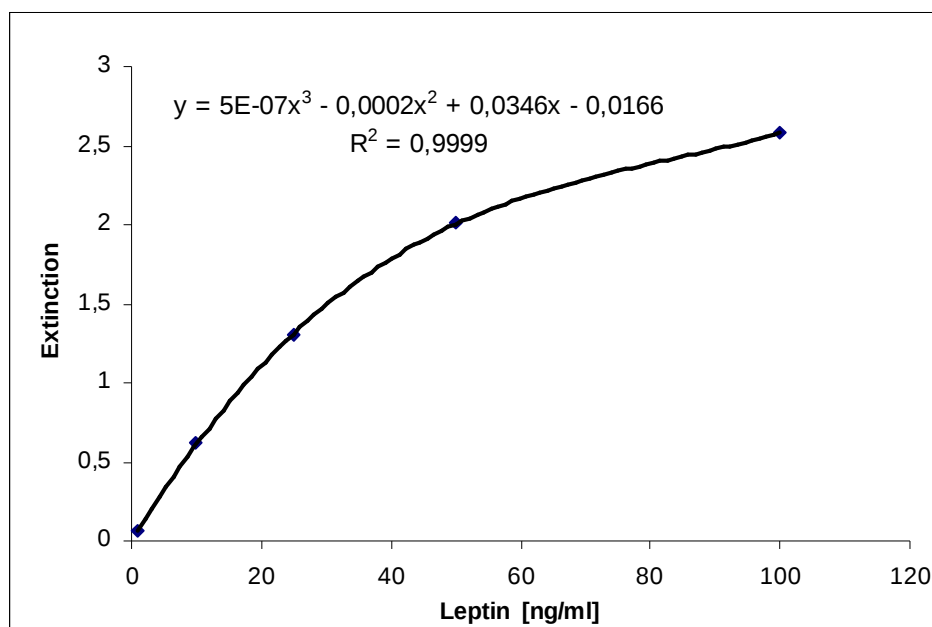
Your **measurement programm** will calculate the leptin concentration of the sample automatically by using the difference of sample and blank for the calculation. You only have to determine the most suitable curve fit (here: polynomial 3<sup>rd</sup> degree).

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

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

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

$$7.012 \text{ ng/mL}$$



**Fig. 1: Exemplary Standard Curve** with a polynomial 3<sup>rd</sup> degree as curve fit.

## 10 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 7 - 14). After the age of 20 years, no significant age dependence was observed. The best-fit regression lines for the various subgroups are exponential curves of the form:

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

**Table 6** 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).

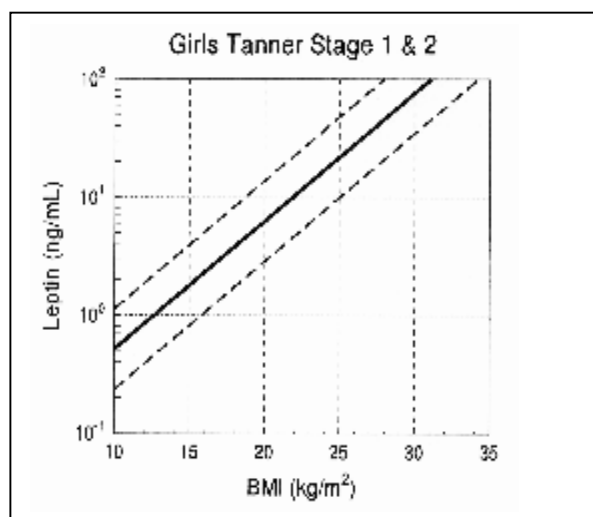
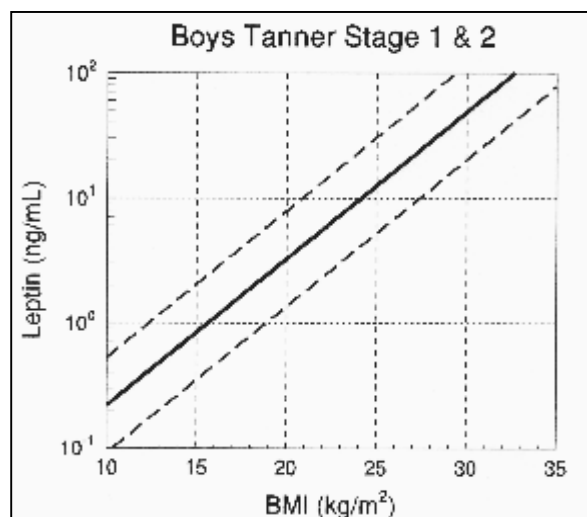
| Cohort          | n   | a      | b      | c      | d      |
|-----------------|-----|--------|--------|--------|--------|
| <b>Males:</b>   |     |        |        |        |        |
| TS 1&2          | 136 | 0,0146 | 0,2706 | 0,8821 | 0,5379 |
| TS 3&4          | 50  | 0,0181 | 0,2067 | 1,1919 | 0,6850 |
| TS 5            | 112 | 0,0316 | 0,1462 | 1,0821 | 0,6558 |
| Adults          | 380 | 0,0130 | 0,2200 | 1,1053 | 0,6740 |
| <b>Females:</b> |     |        |        |        |        |
| 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 7** Girls Tanner stages 1 and 2

| BMI (kg/m <sup>2</sup> ) | Percentile (µg/l) |       |      |       |       |
|--------------------------|-------------------|-------|------|-------|-------|
|                          | 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 8** Boys Tanner stages 1 and 2

| BMI (kg/m <sup>2</sup> ) | Percentile (µg/l) |       |       |       |       |
|--------------------------|-------------------|-------|-------|-------|-------|
|                          | 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             |       |       |       |       |

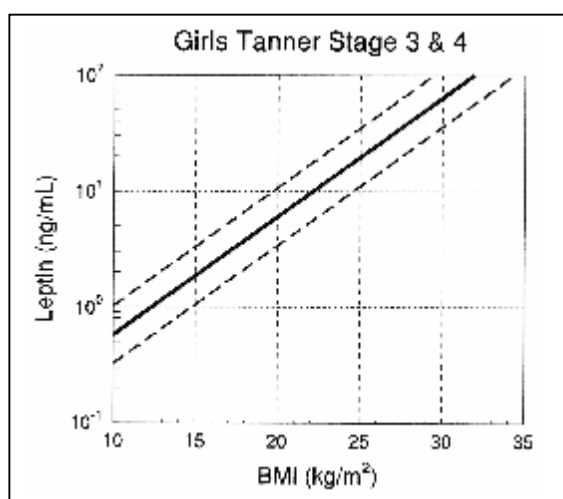
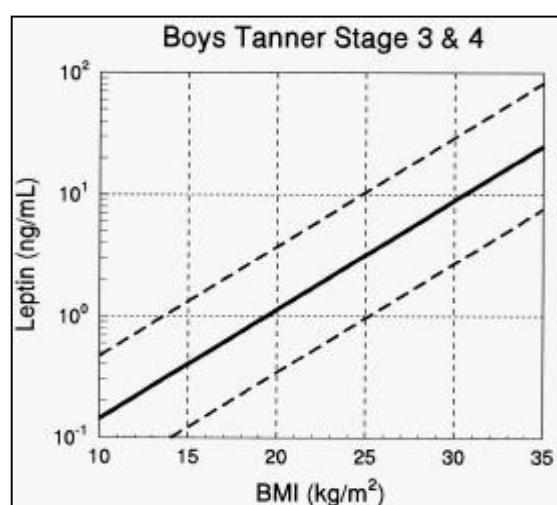
**Figure 2** Exemplary values of human serum levels referring to BMI: Girls Tanner stage 1 & 2 (see text for details).**Figure 3** Exemplary values of human serum levels referring to BMI: Boys Tanner stage 1 & 2 (see text for details).

**Table 9** Girls Tanner stages 3 and 4.

| Percentile (µg/l)        |       |       |       |       |       |
|--------------------------|-------|-------|-------|-------|-------|
| BMI (kg/m <sup>2</sup> ) | 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 |       |       |       |       |

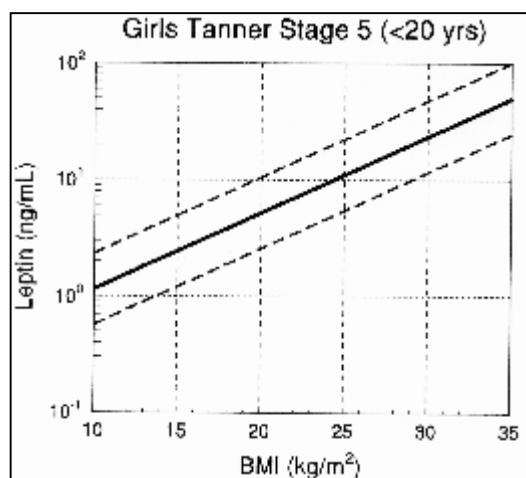
**Table 10** Boys Tanner stage 3 & 4

| Percentile (µg/l)        |      |      |      |       |       |
|--------------------------|------|------|------|-------|-------|
| BMI (kg/m <sup>2</sup> ) | 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 |       |       |

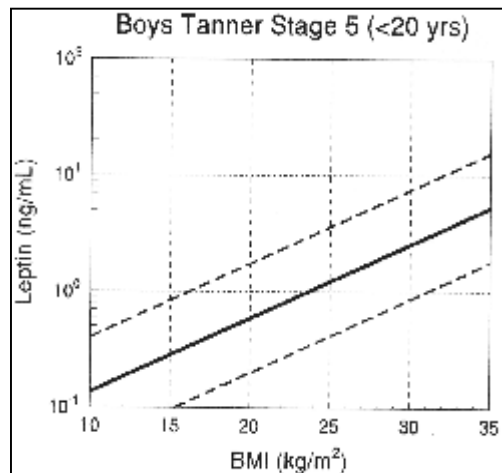
**Figure 4** Exemplary values of human serum levels referring to BMI: Girls Tanner stage 3 & 4 (see text for details).**Figure 5** Exemplary values of human serum levels referring to BMI: Boys Tanner stage 3 & 4 (see text for details).

**Table 11** Girls Tanner stage 5.

| Percentile (µg/l)        |      |      |       |       |       |
|--------------------------|------|------|-------|-------|-------|
| BMI (kg/m <sup>2</sup> ) | 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** Exemplary values of human serum levels referring to BMI: Girls Tanner stage 5 (see text for details).**Table 12** Boys Tanner stage 5

| Percentile (µg/l)        |      |      |      |      |      |
|--------------------------|------|------|------|------|------|
| BMI (kg/m <sup>2</sup> ) | 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 |

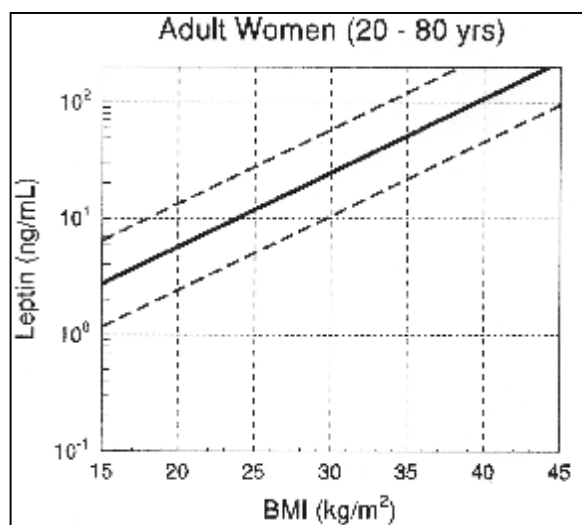
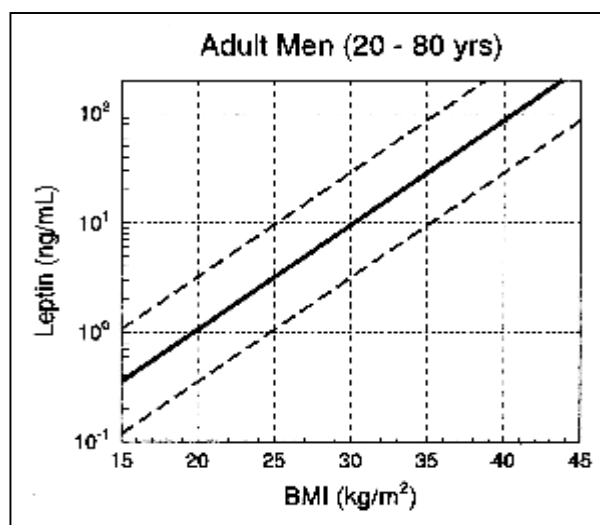
**Figure 7** Exemplary values of human serum levels referring to BMI: Boys Tanner stage 5 (see text for details).

**Table 13** Adult women.

| BMI (kg/m <sup>2</sup> ) | Percentile (µg/l) |      |       |       |       |
|--------------------------|-------------------|------|-------|-------|-------|
|                          | 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 14** Adult men

| BMI (kg/m <sup>2</sup> ) | Percentile (µg/l) |      |      |       |       |
|--------------------------|-------------------|------|------|-------|-------|
|                          | 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** Exemplary values of human serum levels referring to BMI: Adult women (see text for details).**Figure 9** Exemplary values of human serum levels referring to BMI: Adult men (see text for details).

## 11 PERFORMANCE CHARACTERISTICS

### 11.1 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 E07 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.

### 11.2 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<br>[ng/mL] | [n] | Mean<br>[ng/mL] | Standard<br>Deviation<br>[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<br>[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<br>[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<br>[%]       | 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  |

### 11.3 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 Mediagnost Leptin ELISA E07 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           |

### 11.4 Recovery

Recombinant leptin in Dilution Buffer (DIL) 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.

### 11.5 Interference

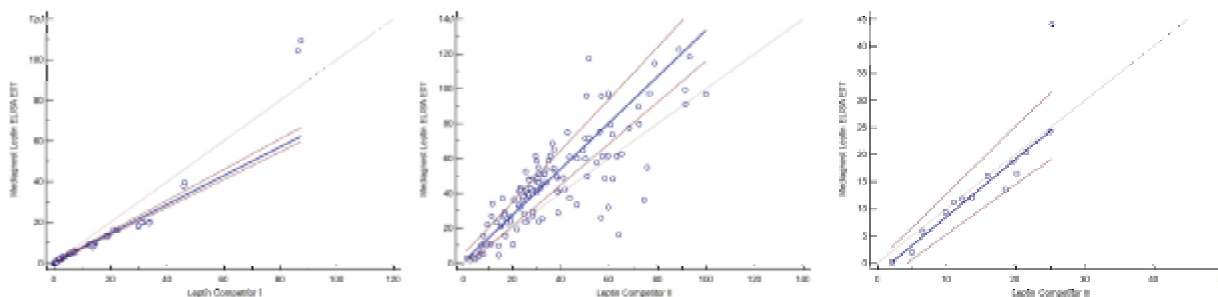
Interference of bilirubin, hemoglobin and triglycerides was tested by adding different amounts of these substances to human serum containing Leptin. For comparison the same amount of buffer without any substance was also added to the serum. Table 14 demonstrates that neither bilirubin nor triglycerides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

**Table 14 Interference of physiological substances**

|         | Triglyceride<br>100 mg/mL | Bilirubin<br>100 µg/mL | Hemoglobin<br>1 mg/mL |
|---------|---------------------------|------------------------|-----------------------|
| Serum 1 | 95                        | 101                    | 94                    |
| Serum 2 | 107                       | 105                    | 105                   |
| Serum 3 | 111                       | 101                    | 101                   |

### 11.6 Comparison Studies

Here the Mediagnost 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 Mediagnost E07. 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)

## 11.7 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.

## 12 Samples other than human serum or plasma

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.

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 considerably, 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 (DIL) + 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           |

## 13 LITERATURE

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## 14 International Assay Description

### International Test Description

|                                                                                                       |                   |                            |
|-------------------------------------------------------------------------------------------------------|-------------------|----------------------------|
| CAL A-E                                                                                               | Rec in 750 µL DIL |                            |
| CTR1                                                                                                  | Rec in 500 µL DIL |                            |
| CTR2                                                                                                  | Rec in 500 µL DIL |                            |
| WB                                                                                                    |                   | 1:20 A. dest.<br>è WB 1:20 |
| -                                                                                                     | SPE               |                            |
| -                                                                                                     | °C 20-25 °C       |                            |
|                                                                                                       |                   |                            |
| 100 µL                                                                                                | DIL               | A1 - End                   |
| 20 µL                                                                                                 | DIL               | 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                                                                                                 | CTR1              | G1/G2                      |
| 20 µL                                                                                                 | CTR2              | H1/H2                      |
| 20 µL                                                                                                 | SPE               |                            |
| TAPE                                                                                                  |                   |                            |
| Å 1 h °C 20-25°C ↔ 200 - 350 rpm                                                                      |                   |                            |
| 5x 300 µL                                                                                             | 5x WB 1:20        |                            |
| 100 µL                                                                                                | DET               |                            |
| TAPE                                                                                                  |                   |                            |
| Å 0.5 h °C 20-25°C ↔ 200 - 350 rpm                                                                    |                   |                            |
| 5x 300 µL                                                                                             | 5x WB 1:20        |                            |
| 100 µL                                                                                                | S                 |                            |
| Å 0.25 h °C 20-25  |                   |                            |
| STP                                                                                                   |                   |                            |
| MEASURE                                                                                               |                   |                            |

**15 ASSAY PROCEDURE**

| Preparation of reagents                                                                  |                                                                                                | Reconstitution                                         | Dilution                          |
|------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|--------------------------------------------------------|-----------------------------------|
| CAL A-E                                                                                  | Calibrators                                                                                    | in 750 µL Dilution Buffer DIL                          | -                                 |
| CTR1 / CTR2                                                                              | Controls                                                                                       | in 500 µL Dilution Buffer DIL                          | -                                 |
| WB                                                                                       | Washing Buffer<br>20-fold conc.                                                                | -                                                      | 1:20 with Aqua dest.<br>è WB 1:20 |
| 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 DIL                                                                            | Pipette in all required wells                          |                                   |
| 20 µL                                                                                    | Dilution Buffer DIL (Blank)                                                                    | A1/A2                                                  |                                   |
| 20 µL                                                                                    | Calibrator A (1 ng/mL)                                                                         | B1/B2                                                  |                                   |
| 20 µL                                                                                    | Calibrator B (10 ng/mL)                                                                        | C1/C2                                                  |                                   |
| 20 µL                                                                                    | Calibrator C (25 ng/mL)                                                                        | D1/D2                                                  |                                   |
| 20 µL                                                                                    | Calibrator D (50 ng/mL)                                                                        | E1/E2                                                  |                                   |
| 20 µL                                                                                    | Calibrator E (100 ng/mL)                                                                       | F1/F2                                                  |                                   |
| 20 µL                                                                                    | Control CTR1 (undiluted)                                                                       | G1/G2                                                  |                                   |
| 20 µL                                                                                    | Control CTR2 (undiluted)                                                                       | H1/H2                                                  |                                   |
| 20 µL                                                                                    | Sample (undiluted) SPE                                                                         | in the rest of the wells<br>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 WB 1:20 / well |                                                        | In each well                      |
| 100 µL                                                                                   | Antibody-POD-Conjugate DET                                                                     |                                                        | 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 WB 1:20 / well |                                                        | In each well                      |
| 100 µL                                                                                   | Substrate S                                                                                    |                                                        | In each well                      |
| Incubation: 15 Minutes in the Dark at 20-25°C                                            |                                                                                                |                                                        |                                   |
| 100 µL                                                                                   | Stop Solution STP                                                                              |                                                        | In each well                      |
| Measure the absorbance within 30 min at 450 nm<br>with ≥ 590 nm as reference wavelength. |                                                                                                |                                                        |                                   |