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Leptin Sensitive ELISA

Enzyme Immunoassay for the high-sensitive quantitative Determination of
Human Leptin
English

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Not for use in diagnostic procedures.

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g



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Manufactured by/ Hergestellt von/ Fabriqué par/ Prodotto da/Fabricado por/ Fabricado por/ Vervaardigd doo/Fabrikation af /Tillverkad av/ Wyprodukowane przez/ Gyártotta / Vyrobené/ Vyrobeno v/ Производител/ Tootja/ Κατασκευάζεται από/ Proodus de/ Proizvajalec/ Valmistaja

h

Catalogue Number/ Bestellnummer/ Numéro de référence/Numero di riferimento/ Número de referencia/ Número de Referência/ Referentienummer/ Referencenummer /Beställningsnummer/ Numer katalogowy/ Rendelési szám/Katalógové číslo/ Objednací číslo/Каталожен номер/Tellimisnumber/ Αρ. παραγγελίας/Număr comandă/ Številka naročila/ viite tai tilausnumero

I

Store at between/ Lagerung bei zwischen/ Conserver à entre/ Conservare a tra/ Conservar a temp. Entre/ Armazenaer 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/ Skladiščenje med/ Säilytys x-y Celsiusasteen lämpötilassa

X

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



Keep away from sunlight/ Nicht dem Sonnenlicht aussetzen/ Conserver à l'abri de la lumière/ Conservare al riparo della luce solare/ No exponer a la luz solar/ Proteger da luz solar/ Niet aan zonlicht blootstellen/ Må ikke udsættes for sollys/ Utsätt inte för solljus/ Nie wystawiać na słońce/ Napfénytől távol tartandó/ Nevystavovat' slnečnému svetlu/ Nevystavovat' slnečnému svétlu/ Да се предпазва от слънчева светлина/ Kaitsta otsese päikesekiirguse eest/ Κρατήστε το μακριά από την ηλιακή ακτινοβολία/ Τίμητε departe de lumina soarelui/ Ne izpostavljajte sončni svetlobi/ suojaa auringonvalolta



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



Shaking/ Schütteln/ Mélanger/ Agitare/ Agitar/ Agitação/ Schudden/ Ryster/ Skaka/ Wstrząsanie/ Rázás/ Pretrepat/ Protřepat/ Разклащане/ Raputada/ Ανακινήστε/ Vibrare/ Stresite/ Sekoita



Mikrotiterplate/ Mikrotiterplatte/ plaque de microtitrage/ Piastra di microtitolazione/ Placa de microtitulación/ Placa de Microtitulação/ microtiterplaat/ Mikrotiterplade/ mikrotiterplatta/ microtiterplaat/ Płytko microtiter/ Mikrotiter lap/ Mikrotitračná podložka/ Mikrotitrační podložka/ Микротитърна плака/ Mikrotiiterplaat/ Τρυβλίο μικροπιλοδότησης/ Microplacă/ Mikrotitrska plošča/ Mikrotittrauslevy



Reconstitute in/ Rekonstituieren in/ Reconstituer dans/ Ricostituire ne/ Reconstituier en/ Reconstituier em/ reconstituieren in/ Rekonstituér i/ rekonstituera/ Rekonstytuować w/ Helyreállítás/ Znovu pripravit' za/ Znovu pripraviti za/ Разтваряне в/ Moodustada uesti / Ανασυστήστε σε/ Reconstituire în/ Predelava v/ rekonstituoi



Sample/ Probe /Echantillon/ campione/ Muestra/ Amostra/ monster/ Prøve/ prov/ Próbka/ Minta/ Vzorka/ Vzorek/ Проба/ Proov/ Δείγμα/ Probã/ Vzorec/ Näyte



AK Antibody and Enzyme Conjugate/ Antikörper und Enzym Konjugat/ anticorps conjuguée et conjugué enzymatique/ Coniugato di anticorpo ed enzima/ Conjugado de anticuerpos y enzimas/ Conjugado Anticorpo-Enzima/ antilichaamen enzymconjugaat/ Antistoffer og enzym-konjugat/ antikropps- och enzymkonjugat (antikropp och enzym, konjugat)/ Koniugat antycial i enzymów/ Antitest és enzim páros/ Protílatkový a enzymatický konjugát/ Protílatkový a enzymatický konjugát/ Антитяло и ензим конюгат/ Antikehad ja ensüümi konjugaat/ Σύμπλοκο αντισώματος-ενζύμου/ Compuși din anticorpi și enzime/ Antitelesa in konjugat encima/ Vasta-aine ja entsymi konjugaatti



VP Buffer / puffer/ Tampon / Tampone / Tampón/Tampão / buffer/ buffer/ buffer/ Bufor / puffer/ pufer/ pufr/ буфер/ puhver/ ρυθμιστικό διάλυμα / Tampon /pufer/ puskuriliuos

| | | | |
|----------------------|------------------------------------|--------------------|---|
| DILU | BUF | | Dilute in Buffer X/ Verdünnen in Puffer X/ Diluer dans le tampon X/ Diluire nel tampone X/ Diluir en tampón X/ Diluir no Tampão X/ verdunnen in buffer X/ Fortyndes i buffer X/ späd i buffert X/ Rozcieńczanie w buforze X/ Hígítás X pufferben/ Riedit' v pufrí X/ Ředit v pufru X/ Разреждане в буфер X/ Lahjendada puhvrís X/ Αραιώστε σε ρυθμιστικό διάλυμα X/ Diluați în tamponul X/ Razredčiti v pufru X/ Iaimennetaan x puskuriin |
| | X | | |
| | STD | X | A-E Standard X/ Standard X/ Etalon X/ Standard X/ Estándar X/ Standard X/ standaard X/ Standard X/ standard X/ Standard X/ Standard X/ Štandard X/ Standard X/ Стандарт X/ Standard X/ Πρότυπο X/ Standard X/ Standardni X/ Kalibraattori X |
| | Control | | KS1/ Control Serum / Kontrollserum/ Contrôle sérique/ Siero di controllo/ Suero de control/ Soro de Controlo/ KS2 controleserum/ Kontrollserum/ Kontrollserum/ Serum kontrolne/ Ellenőrző szérum/ Kontrollné sérum/ Kontrolní sérum/ Контролен серум/ Kontrollseerum/ Ορός ελέγχου/Ser de control/ Kontrolni serum/ Kontrolli seerumi |
| | WASHBUF | 20x | WP Washing Buffer Concentrate/ Waschpufferkonzentrat/ Tampon de lavage conc./ Tampone di lavaggio concentrato/ Tampón de lavado concentrado/ Tampão de Lavagem Concentrado/ wasbuffer, geconcentreerd/ Vaskebufferkonzentrat/ Vaskebufferkonzentrat/ tvättbuffertkonzentrat/ Bufor płukania koncentrat/ Mosópuffer koncentrátum/ Koncentrát vymývacieho pufru/ Концентрат на промивен буфер/ Pesupuhvri kontsentraat/ Συμπύκνωμα ρυθμιστικού διαλύματος έκπλυσης/ Concentrat pentru tamponul de spălare/ Koncentrat izpiralnega pufru/ Pesuliuositiiviste |
| | WASHBUF | | Washing Buffer / Waschpuffer/ Tampon de lavage/ Tampone di lavaggio/ Tampón de lavado/Tampão de Lavagem/ wasbuffer/ Vaskebuffer/ tvättbuffert/ Bufor płukania/ Mosópuffer/ Vymývací pufer/ Vymývací pufr/ Προμивен буфер/ Pesupuhver/ Ρυθμιστικό διάλυμα έκπλυσης/ Tampon pentru spălare /Izpiralni pufer/ Pesuliuos |
| | SUBST | TMB | S Substrate/ Substrat/ Substrat/ Substrato/ Substrato/ Substrato/ substraat/ Substrat/ Substrat/ Substrat/ Substrat/ Substratum/ Substrát/ Substrát/ Субстрат Substraat/ Υπόστρωμα/ Substrat/ Substrat/ Substraattiliuos |
| | H₂SO₄ | | SL Stop Solution/ Stopp Lösung/ Stop Solution/ Soluzione di stop/ Stop Solución/ Solução Stop/ stopoplossing/ Stopopløsnng/ Stoppløsning/ Stop roztwór/ Megállító oldat/ Roztok na ukončenie/ Roztok pro ukončeni/ Стопираш разтвор/ Stopp-lahus/ Διάλυμα διακοπής/ Soluție de oprire/ Stop raztopina/ Pysäytysliuos |
| | TAPE | | Cover Plate with sealing tape /Platte abkleben/ Recouvrir la microplaque avec bande adhésive/ Coprire la piastra con nastro adesivo/ Cubrir la placa con una cinta adhesiva/ Cobrir a Placa com fita adesiva/ plaatje met tape afdekken/ Afdækningsplade med tape/ maskera platta/ Odkleić plytkę/ Tányér leragasztása/ Oblepiť podložku lepiacou páskou/ Olepit podložku lepící páskou/ Плака с лента за запечатване/ Katta plaat isoleerkiieplindiga/ Κολλήστε το πλακίδιο με κολλητική ταινία/ Aoperiți placa cu o bandă adezivă/ Prelepiti ploščo/ Peitã mikrotitrauslevyn oheisella teipillä |
| MEASURE | | | Measure plate within 30 min at 450 nm (Referencefilter ≥590nm)/Ausmessung innerhalb von 30 min bei 450 nm (Referenzfilter ≥ 590 nm)./ Mesure lábsorbance en léspace de 30 min à450 nm avec ≥590nm longueur dónce pour référence/Misurazione entro 30 min. a 450 nm (filtro di riferimento ≥ 590 nm)./ Medición de la placa dentro de los siguientes 30 min a 450 nm (filtro de referencia ≥ 590nm)/ Medir a placa dentro de 30 min a 450 nm (Filtro de referência ≥590nm)/ Binnen 30 minuten bij 450 nm meten (referentiefilter ≥ 590 nm)./ Mål plade i løbet af 30 min ved nm (referencefilter ≥590nm)/ Mät inom 30 min vid 450 nm (referensfilter ≥ 590 nm)./ Pomiar w ciągu 30 min przy 450 nm (filtr odniesienia ≥ 590 nm)./ Ki mérés 30 percen belül 450 nm-nél (referenciaszűrő ≥ 590 nm)./ Merať 30 minút pri 450 nm/Měřit 30 minut při 450 nm/ Отчитане в рамките на 30 min при 450 nm (референтен филтър ≥ 590 nm)./ Mõõtmise 30 min jooksul 450 nm korral (võrdlusfilter ≥ 590 nm). Μέτρηση εντός 30 min στα 450 nm (φίλτρο αναφοράς ≥ 590 nm)./ Măsurare în decurs de 30 min la 450 nm (filtru de referință ≥ 590 nm)./ Izmerite ploščico v 30 min pri 450 nm (referenčni filter ≥590nm) / Mittaa 30 minuutin aikana 450 nm:ssä (referenssi suodatin ≥ 590 nm) |
| Literatur | | | Literature/ Literatur/ Bibliographie/ Letterario/ Bibliografia/ Literatura documentação/ literatuur/ Litteratur/ Literatur/ Literatura/ Irodalom/ Literatúra/ Literatura/ Литература// Kirjandus/ Βιβλιογραφία/ Bibliografie/ literatura/ Lähdeluettelo |
| International | Test | description | International test description/ internationale Testanleitung/ description internationale de test/ Istruzioni per il test internazionali/ Descripción de ensayo internacional/ Descrição internacional do teste/ internationale testbeschrijving/ internationell testbeskrivning/ Opis testu międzynarodowego/ nemzetközi teszt-útmutató/ Medzinárodný návod k testu/ Mezinárodní návod k testu/ rahvusvaheline katse kirjeldus/ Διεθνείς οδηγίες για εργαστηριακές δοκιμές/ instrucțiuni internaționale pentru testare/ mednarodna navodila za preizkus/ Kansainvälinen käyttöohje |
| End | | | in all required wells/ in allen benötigten Vertiefungen/ dans tous les godets requis/ in tutti i pozzetti richiesti/ en todos los pozos requeridos/ em todos os tubos necessários/ in alle nodige putjes/ i alle nødvendige brønde/ i alla nödvändiga brunnar/ we wszystkich potrzebnych wgłębieniach/ minden szükséges forrásban/ vo všetkých potrebných miestach/ ve všech potřebných místech/ във всички необходими ямки/ kõigis vajalikes süvendites/ σε όλες τις απαραίτητες κοιλότητες/ în toate cavitățile necesare/ v vseh zahtevanih vdolbinah /kaikkiin tarvittaviin mikrotitrauslevyn syvennyksiin |

For Research Use Only.

Not for use in diagnostic procedures.

CAUTION: Not for human or animal therapeutic or diagnostic use.

For in vitro use only.

For professional use only.

Read entire protocol before use!

| | |
|--|----|
| Symbols/ Symbole /Symboles/ Simboli/ Símbolos/ Símbolos/ Symbolen/ Symboler/ Symboler/ Symbole/ Szimbólumok/ Symboly/ Symboly/ Символи/ Sümbolid/ Σύμβολα/ Simboluri/ Simboli/ Symbolit | 2 |
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Mediagnost human Leptin SENSITIVE ELISA E077

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

INTENDED USE

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

INTRODUCTION

Leptin, the product of the ob gene (1,2), is a recently discovered single-chain proteohormone with a molecular weight of 16 kD, which is thought to play a key role in the regulation of body weight. Its amino acid sequence exhibits no major homologies with other proteins (1). Leptin is almost exclusively produced by differentiated adipocytes (3-5). It acts on the central nervous system, in particular the hypothalamus, thereby suppressing food intake and stimulating energy expenditure (2,6-9). Leptin receptors - alternatively spliced forms exist that differ in length - belong to the cytokine class I receptor family (10-12). They are found ubiquitously in the body (10, 11, 13, 14) indicating a general role of leptin, which is currently not fully understood. A circulating form of the leptin receptor exists which acts as one of several leptin binding proteins (15). Besides its metabolic effects, leptin was shown to have a strong influence on a number of endocrine axes. In male mice, it blunted the starvation-induced marked decline of LH, testosterone, thyroxine and the increase of ACTH and corticosterone. In female mice, leptin prevented the starvation-induced delay in ovulation (16). Ob/ob mice, which are leptin deficient due to an ob gene mutation, are infertile. This defect could be corrected by administration of leptin, but not through weight loss due to fasting (17), suggesting that leptin is pivotal for reproductive functions.

All these actions may, at least in part, be explained by the suppressive effect of leptin on neuropeptide Y (NPY) expression and secretion by neurons in the arcuate nucleus (6,18,19). NPY is a strong stimulator of appetite (20,21) and is known to be involved in the regulation of various pituitary hormones, e.g. suppression of GH through stimulation of somatostatin (22,23), suppression of gonadotropins (23) or stimulation of the pituitary-adrenal axis (21).

The most important variable that determines circulating leptin levels is body fat mass (24-26). Obviously, under conditions of regular eating cycles, leptin reflects the proportion of adipose tissue (27) showing an exponential relationship (37). This constitutive synthesis of leptin is modulated by a number of non-hormonal and hormonal variables. Stimulators in both rodents and humans are overfeeding (28,29), insulin (3,5,30-33) and glucocorticoids (5,34-36). Suppression has been shown for fasting (27), cAMP and beta-3-adrenoceptor agonists (35).

From these findings it becomes clear that leptin is an integral component of various metabolic and endocrine feedback loops (38).

Serum leptin levels show a moderate **circadian variation** with a peak during the night at about 2 a.m. (37). The leptin values at this time are about 30 to 100 % higher than the levels measured in the morning or early afternoon. This variation together with the influence of food intake needs to be taken into account, when blood samples are collected.

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

REAGENTS PROVIDED

| | | |
|----|-------------------------|--|
| 1) | MTP | Microtiter plate , ready for use: Microtiter plate with 96 wells, divided up in 12 strips with 8 wells separately breakable, coated with antibody against leptin, packed in a laminate bag. |
| 2) | STD | Standards A-E, 1 ml lyophilized: Contain recombinant Leptin and have to be reconstituted with 1 ml Dilution Buffer VP each. After using store the reconstituted standards in the original flasks as soon as possible at -20°C . When using the standards anew, please thaw them rapidly but gently (no temperature rise over the room temperature and no powerful vortexing), 3 of these freezing-thawing cycles showed no influence on the assay. Standard values are between 0.05 – 5 ng/ml (0.05; 0.5; 1.5; 3.5; 5 ng/ml) recombinant human leptin |
| 3) | BUF | Dilution Buffer VP, 60 ml , ready for use, please use for the reconstitution of Standards A-E, Control Sera KS1 & KS2. |
| 4) | Control | Control Sera KS1 & KS2, each 250 μl lyophilized: Contains human serum. Reconstitute each with 250 μl Dilution Buffer VP . Exact human leptin concentration and the acceptable range are given on the certificate. The reconstituted Control Sera must be stored in the original flask as soon as possible at -20°C after use. When using anew, please thaw it rapidly but gently (no temperature rise over the room temperature and no powerful vortexing), 3 of these freezing-thawing cycles showed no influence on the assay. |
| 5) | Ab CONJ | anti-Leptin Antibody POD-Conjugate AK, 12 ml , ready for use: Contains a mix of biotinylated anti-human leptin antibody and Horseradish peroxides conjugated streptavidin. Use 100 μl per well in the assay |
| 6) | WASHBUF 20x | Washing Buffer (WP), 50 ml , 20 X concentrated solution. Washing Buffer (WP) has to be diluted 1:20 with distilled or demineralised water before use (e.g. add the complete contents of the flask (50 ml) into a graduated flask and fill up with A.dest. to 1000 ml). Attention: After dilution the Washing Buffer is only 4 weeks stable, dilute only according to requirements. |
| 7) | SUBST | Substrate (S), 12 ml , ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbencidine. |
| 8) | H_2SO_4 | Stopping Solution (SL), 12 ml , ready for use, 0.2 M sulphuric acid, Caution acid! |
| 9) | | Sealing tape for covering of the microtiter plate, 2 x, adhesive. |

MATERIALS REQUIRED BUT NOT PROVIDED

Precision pipettes, Micropipettes and multichannel pipettes with disposable plastic tips

Distilled or deionized water for dilution of the Washing Buffer (WP)

Vortex-mixer

Microtiter plate shaker (350 rpm)

Microtiter plate washer (recommended)

Micro plate reader ("ELISA-Reader") with filter for 450 and ≥ 590 nm

Polyethylene PE/Polypropylene PP tubes for dilution of samples

WARNINGS AND PRECAUTIONS

For research and professional use only.

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

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

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

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

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

| | |
|--------|--|
| R34 | Irritating to eyes and skin |
| R43 | Sensibilisation through skin contact possible |
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice |
| S36/37 | Wear suitable protective clothing and gloves |
| S45 | In case of accident or if you feel unwell seek medical advice |

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

| | |
|-------------|--|
| R36/38 | Irritating to eyes and skin |
| R43 | Sensibilisation through skin contact possible |
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice |
| S28.1 S28.1 | After contact with skin, wash immediately with plenty of water |

Stop solution contains 0.2 M Sulfuric Acid (H₂SO₄)

| | |
|--------|--|
| R36/38 | Irritating to eyes and skin |
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice |
| S28.1 | After contact with skin, wash immediately with plenty of water |
| S36/37 | Wear suitable protective clothing and gloves. |

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

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

| | |
|------------|--|
| R20/21/R22 | Harmful by inhalation, in contact with skin and if swallowed |
| R36/37/38 | Irritating to eyes, respiratory system and skin |
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice |
| S28.1 | After contact with skin, wash immediately with plenty of water |
| S36/37 | Wear suitable protective clothing and gloves |

General first aid procedures:

Skin contact: Wash affected area thoroughly with water. Discard contaminated cloths and shoes.

Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing spread the eyelids.

Ingestion: If swallowed, wash out mouth thoroughly with water. Immediately see a physician.

Do not eat, drink or smoke in these areas.

Never pipette the materials with the mouth.

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

PRINCIPLE

The Mediagnost ELISA for Leptin E077 is a so-called Sandwich-Assay using two specific and high affinity antibodies. The Leptin in the samples binds to the first antibody coated on the 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.

SPECIMEN

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

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

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

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

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

Storage of the samples

Storage at RT max. 2 days

Storage at -20°C max. 2 years

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

Sample Preparation

Samples have to be diluted in Dilution Buffer (VP).

We recommend a standard dilution on 1:10.

Suggestion for dilution protocol:

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

If Leptin levels over 50 ng/ml are expected, the sample should be diluted higher in Dilution Buffer VP, e.g. 1:20.

The hLeptin concentrations may be completely different in body fluids of human origin other than serum or cell culture supernatants. In samples of urine or breast milk very low levels were determined. Furthermore this kind of samples must be diluted at least 1:5. If samples are used undiluted, the reaction will be interfered and the values are reduced (s. Assay Characteristics and Validation).

TECHNICAL NOTES

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

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

The shelf life of the components after opening is not affected, if used appropriately.

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming.

Incubation at room temperature means: 20-25°C

The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must become adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/or false values, excessive shaking may result in high optical densities and/or false values.

Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems.

All washing must be performed with the provided washing buffer diluted to usage concentration. Washing volume per washing cycle and well must be 300 µl at least.

The danger of handling with potentially infectious material must be taken into account. When using an automatic 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 dynamically 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.

Standards and Controls

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

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

Washing Buffer

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

Microtiterplate

Unused microtiterplate stripes have to be stored airtight together with the desiccant bag at $2-8^{\circ}\text{C}$. The labelled expiry is not influenced in case of proper storage.

ASSAY PROCEDURE

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

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

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

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

- 1) add **100 μl** Dilution Buffer VP to the first wells (blank). Subsequently, add 100 μL of each Standard or diluted Control (KS1&KS2) or diluted Sample to the following wells.
- 2) cover the wells with sealing tape and incubate the plate for **1 hour** shaking with **350 rpm**.
- 3) after incubation aspirate the contents of the wells into a disinfectant (possible theoretically risk of infection!) and wash the wells **3 times** with **300 μl** of **Washing Buffer WP** / well respectively. The washing buffer WP should incubate at least for 15 seconds/cycle
- 4) pipette **100 μl** of the **Antibody-POD-Conjugate AK** in each well and incubate **30 minutes** shaking with **350 rpm**.
- 5) after incubation wash the wells 3 times with Washing Buffer as described in step 3
- 6) pipette **100 μL** of the **Substrate (S)** in each well.
- 7) incubate the plate for **15 minutes in the dark at room temperature (20 - 25°C)**.
- 8) stop the reaction by adding **100 μL** of **Stopping Solution (SL)**.
- 9) measure the colour reaction within 30 minutes at **450 nm** (reference filter $\geq 590 \text{ nm}$).

CALCULATION OF RESULTS

Establishing the Standard Curve

For the evaluation of the assay it is preconditioned that the absorbance values of the blank should be below 0.25, these of standard E should be above 1.0.

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

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

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

- 1) Calculate the **mean absorbance** value for the blank from the duplicated determination (well A1/A2).
- 2) Subtract the mean absorbance of the blank from the mean absorbances of all other values.
- 3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
- 4) Recommendation: Calculation of the standard curve should be done by using a computer program. **A higher-grade polynomial, or four parametric logistic (4-PL) curve fit or non-linear regression** are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).
- 5) The **concentration in ng/ml** of the samples can be calculated **by multiplication** with the respective dilution factor.

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

Exemplary calculation of the leptin concentration of an undiluted sample:

Measured extinction of your sample 0.293

Measured extinction of the blank 0.015

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

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

$$\begin{aligned} 0.278 &= -0.0089658 + 0,64743x - 0.034025x^2 \\ 0.4524 &= x \end{aligned}$$

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

$$0.4524 \times 10 \text{ ng/mL} = 4.524 \text{ ng/mL}$$

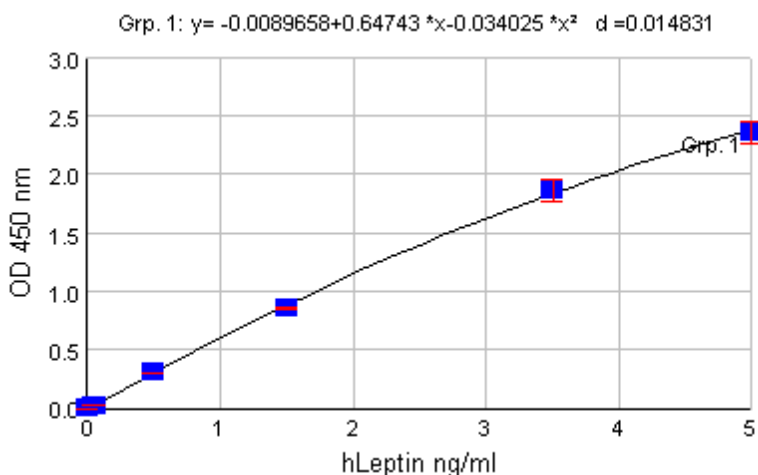


Fig. 1: Exemplary Standard Curve with a polynomial 2nd degree as curve fit.

PERFORMANCE CHARACTERISTICS

Standards

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

Sensitivity

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

Specificity

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

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

Reproducibility and Precision

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

Table 1 : Inter-Assay-Variation

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

Table 2: Intra-Assay-Variation

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

EXEMPLARY VALUES

Serum leptin levels are mainly determined by body fat mass with low levels in lean individuals and high levels in obese subjects. In addition, there is a clear gender difference with higher levels in females at a given percentage body fat. Further, leptin levels are influenced by pubertal development. Any attempt, therefore, to give ranges of expected leptin levels must account for these relationships.

The following exemplary serum leptin levels were referred to BMI as the major confounding independent variable and were stratified according to gender and pubertal development (45; see figures 2-9 and tables 6 - 13). After the age of 20 years, no significant age dependence was observed. These. The best-fit regression lines for the various subgroups are exponential curves of the form:

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

Appendix /Anhang

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

| Cohort | n | a | b | c | d |
|-------------------|-----|--------|--------|--------|--------|
| Males: | | | | | |
| TS 1&2 | 136 | 0,0146 | 0,2706 | 0,8821 | 0,5379 |
| TS 3&4 | 50 | 0,0181 | 0,2067 | 1,1919 | 0,6850 |
| TS 5 | 112 | 0,0316 | 0,1462 | 1,0821 | 0,6558 |
| Adults | 380 | 0,0130 | 0,2200 | 1,1053 | 0,6740 |
| Females | | | | | |
| TS 1&2 | 136 | 0,0422 | 0,2499 | 0,7849 | 0,4786 |
| TS 3&4 | 43 | 0,0543 | 0,2357 | 0,5745 | 0,3379 |
| TS 5 | 157 | 0,2550 | 0,1508 | 0,7053 | 0,4301 |
| Adults | 587 | 0,3042 | 0,1467 | 0,8548 | 0,5212 |

Table 4: Girls Tanner stages 1 and 2

| Percentile / Perzentile (µg/L) | | | | | |
|--------------------------------|------|------|------|-------|-------|
| BMI (kg/m ²) | 1 | 5 | 50 | 95 | 99 |
| 11 | 0.22 | 0.30 | 0.66 | 1.45 | 1.99 |
| 12 | 0.28 | 0.39 | 0.85 | 1.86 | 2.56 |
| 13 | 0.36 | 0.50 | 1.09 | 2.38 | 3.29 |
| 14 | 0.46 | 0.64 | 1.40 | 3.06 | 4.22 |
| 15 | 0.60 | 0.82 | 1.79 | 3.93 | 5.42 |
| 16 | 0.76 | 1.05 | 2.30 | 5.04 | 6.96 |
| 17 | 0.98 | 1.35 | 2.95 | 6.47 | 8.93 |
| 18 | 1.25 | 1.73 | 3.79 | 8.31 | 11.5 |
| 19 | 1.61 | 2.22 | 4.87 | 10.7 | 14.7 |
| 20 | 2.07 | 2.85 | 6.25 | 13.7 | 18.9 |
| 21 | 2.65 | 3.66 | 8.03 | 17.6 | 24.3 |
| 22 | 3.41 | 4.70 | 10.3 | 22.6 | 31.2 |
| 23 | 4.37 | 6.03 | 13.2 | 29.0 | 40.0 |
| 24 | 5.62 | 7.75 | 17.0 | 37.2 | 51.4 |
| 25 | 7.21 | 9.95 | 21.8 | 47.8 | 65.9 |
| 26 | 9.26 | 12.8 | 28.0 | 61.4 | 84.7 |
| 27 | 11.9 | 16.4 | 35.9 | 78.8 | 109.0 |
| 28 | 15.3 | 21.1 | 46.1 | 101.0 | 140.0 |
| 29 | 19.6 | 27.0 | 59.2 | 130.0 | |
| 30 | 15.2 | 34.7 | 76.1 | | |
| 31 | 32.3 | 44.6 | 97.7 | | |
| 32 | 41.5 | 57.2 | 125. | | |
| 33 | 53.2 | 73.4 | | | |
| 34 | 68.4 | 94.3 | | | |
| 35 | 87.8 | 121. | | | |
| 36 | 113 | | | | |
| 37 | 145 | | | | |

Table 5: Boys Tanner stages 1 and 2

| Percentile / Perzentile (µg/L) | | | | | |
|--------------------------------|-------|-------|-------|-------|-------|
| BMI (kg/m ²) | 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 | | | | |

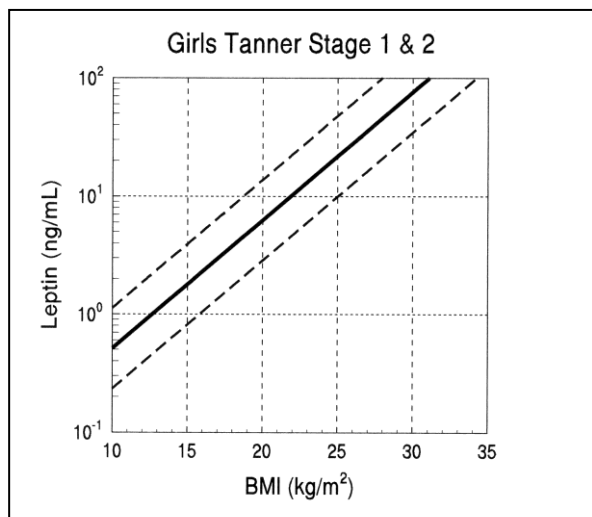


Figure 2: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 1 & 2 (see text for details)

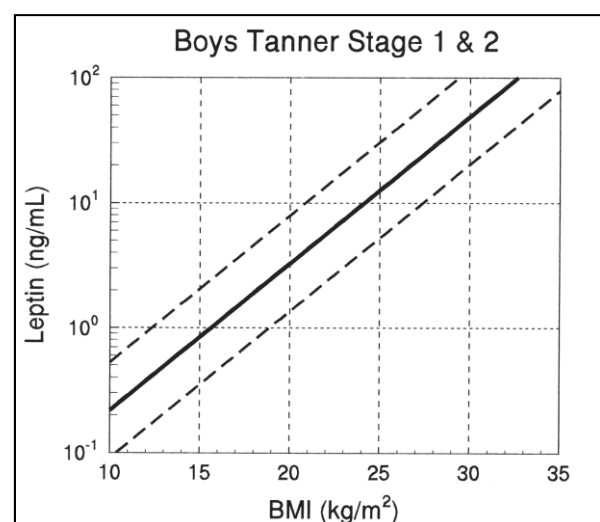


Figure 3: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 1 & 2 (see text for details)

Table 6: Girls Tanner stages 3 and 4.

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

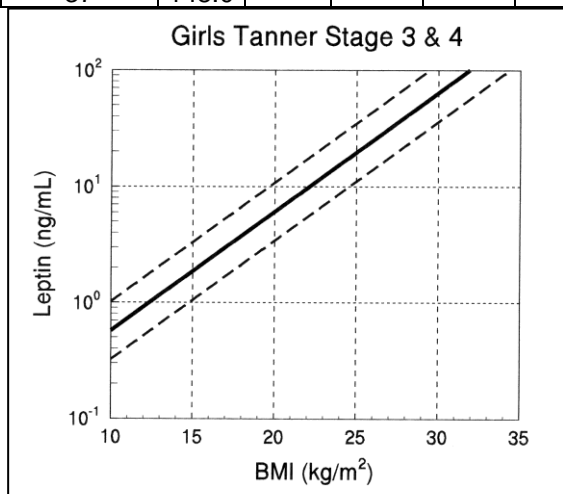


Figure 4: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 3 & 4 (see text for details).

Table 7 : Boys Tanner stage 3 & 4

| Percentile / Perzentile (µg/L) | | | | | |
|--------------------------------|------|------|------|-------|-------|
| BMI (kg/m ²) | 1 | 5 | 50 | 95 | 99 |
| 11 | 0.03 | 0.05 | 0.18 | 0.58 | 0.94 |
| 12 | 0.04 | 0.07 | 0.22 | 0.71 | 1.16 |
| 13 | 0.49 | 0.08 | 0.27 | 0.88 | 1.43 |
| 14 | 0.06 | 0.10 | 0.33 | 1.08 | 1.75 |
| 15 | 0.07 | 0.12 | 0.40 | 1.32 | 2.16 |
| 16 | 0.09 | 0.15 | 0.49 | 1.63 | 2.65 |
| 17 | 0.11 | 0.18 | 0.61 | 2.00 | 3.26 |
| 18 | 0.14 | 0.23 | 0.75 | 2.46 | 4.01 |
| 19 | 0.17 | 0.28 | 0.92 | 3.03 | 4.93 |
| 20 | 0.21 | 0.34 | 1.13 | 3.72 | 6.06 |
| 21 | 0.26 | 0.42 | 1.39 | 4.58 | 7.46 |
| 22 | 0.32 | 0.52 | 1.71 | 5.63 | 9.17 |
| 23 | 0.39 | 0.64 | 2.10 | 6.92 | 11.3 |
| 24 | 0.48 | 0.78 | 2.58 | 8.51 | 13.9 |
| 25 | 0.59 | 0.96 | 3.18 | 10.5 | 17.0 |
| 26 | 0.73 | 1.19 | 3.91 | 12.9 | 21.0 |
| 27 | 0.89 | 1.46 | 4.80 | 15.8 | 25.8 |
| 28 | 1.10 | 1.79 | 5.90 | 19.4 | 31.7 |
| 29 | 1.35 | 2.20 | 7.26 | 23.9 | 39.0 |
| 30 | 1.66 | 2.71 | 8.93 | 29.4 | 48.0 |
| 31 | 2.05 | 3.33 | 11.0 | 36.2 | 58.9 |
| 32 | 2.51 | 4.09 | 13.5 | 44.5 | 72.4 |
| 33 | 3.09 | 5.04 | 16.6 | 54.7 | 89.1 |
| 34 | 3.80 | 6.20 | 20.4 | 67.2 | 109.0 |
| 35 | 4.68 | 7.62 | 25.1 | 82.6 | 134.0 |
| 36 | 5.75 | 9.37 | 30.9 | 101.0 | |
| 37 | 7.07 | 11.5 | 37. | 124.0 | |
| 38 | 8.7 | 14.2 | 46.7 | | |
| 39 | 10.7 | 17.4 | 57.4 | | |
| 40 | 13.1 | 21.4 | 70.5 | | |

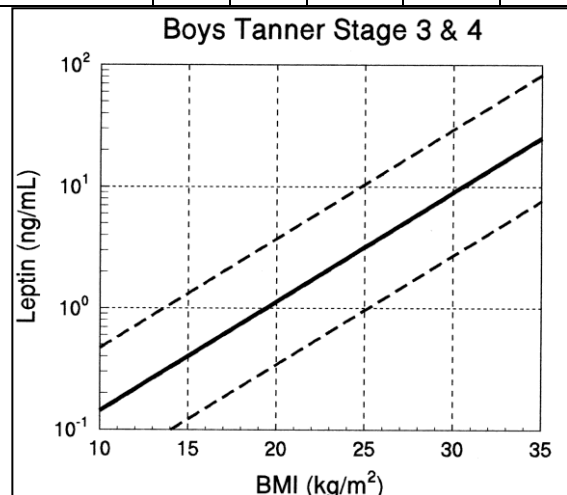


Figure 5: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 3 & 4 (see text for details).

Table 8 : Girls Tanner stage 5.

| Percentile /Perzentile (µg/L) | | | | | |
|-------------------------------|------|------|-------|-------|-------|
| BMI (kg/m ²) | 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 9 : Boys Tanner stage 5

| Percentile / Perzentile (µg/L) | | | | | |
|--------------------------------|------|------|------|------|------|
| BMI (kg/m ²) | 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 |

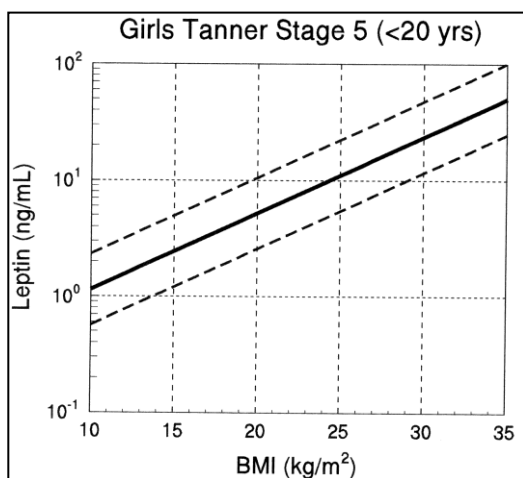


Figure 6: Reference ranges of human serum levels referring to BMI: Girls Tanner stage 5 (see text for details)

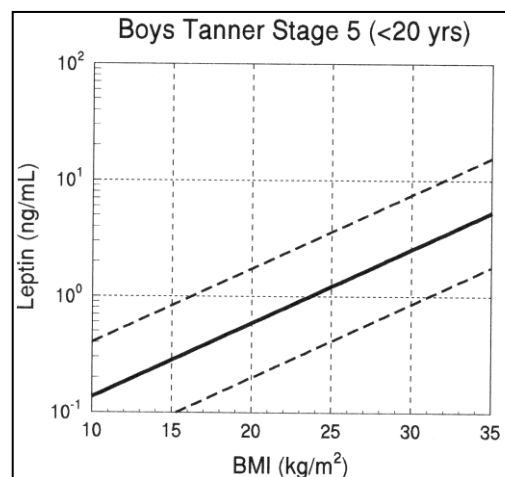


Figure 7: Reference ranges of human serum levels referring to BMI: Boys Tanner stage 5 (see text for details).

Table 10: Adult women.

| Percentile / Perzentile (µg/L) | | | | | |
|--------------------------------|------|------|-------|-------|-------|
| BMI (kg/m ²) | 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 11 Adult men

| Percentile / Perzentile (µg/L) | | | | | |
|--------------------------------|------|------|------|-------|-------|
| BMI (kg/m ²) | 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 | | |

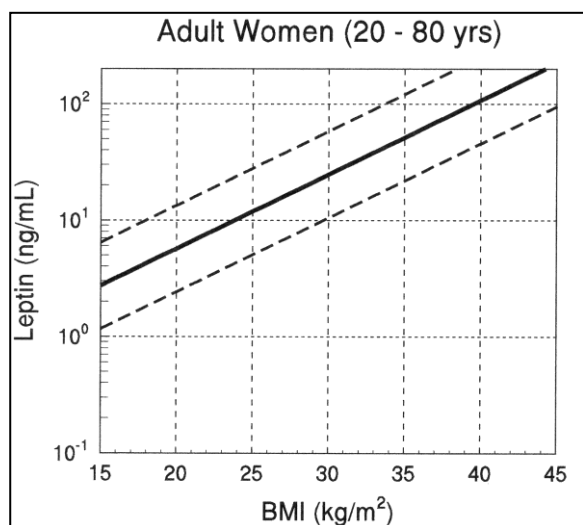


Figure 8: Reference ranges of human serum levels referring to BMI: Adult women (see text for details)

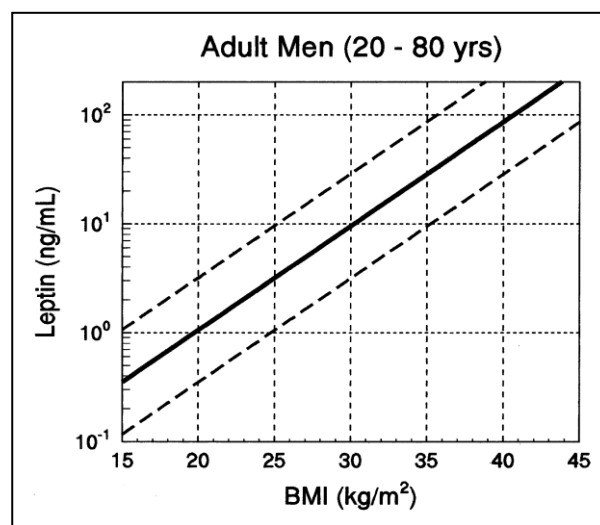


Figure 9: Reference ranges of human serum levels referring to BMI: Adult men (see text for details).

LITERATUR / LITERATURE

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature*. 372:425-432.
2. Halaas JL, Gajiwala KS, Maffei M, et al. 1995 Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*. 269:543-546.
3. MacDougald OA, Hwang CS, Fan H, Lane MD. 1995 Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A*. 92:9034-9037.
4. Rentsch J, Chiesi M. 1996 Regulation of ob gene mRNA levels in cultured adipocytes. *FEBS Lett*. 379:55-59.
5. Wabitsch M, Jensen PB, Blum WF, et al. 1996 Insulin and cortisol promote leptin production in cultured human fat cells. *Diabetes*. 45:1435-1438.
6. Stephens TW, Basinski M, Bristow PK, et al. 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature*. 377:530-532.
7. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. 1995 Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science*. 269:546-549.
8. Pelleymounter MA, Cullen MJ, Baker MB, et al. 1995 Effects of the obese gene product on body weight regulation in ob/ob mice. *Science*. 269:540-543.
9. Levin N, Nelson C, Gurney A, Vandlen R, de-Sauvage F. 1996 Decreased food intake does not completely account for adiposity reduction after ob protein infusion. *Proc Natl Acad Sci U S A*. 93:1726-1730.
10. Tartaglia LA, Dembski M, Weng X, et al. 1995 Identification and expression cloning of a leptin receptor, OB-R. *Cell*. 83:1263-1271.
11. Chen H, Charlat O, Tartaglia LA, et al. 1996 Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell*. 84:491-495.
12. Lee GH, Proenca R, Montez JM, et al. 1996 Abnormal splicing of the leptin receptor in diabetic mice. *Nature*. 379:632-635.
13. Lynn RB, Cao GY, Considine RV, Hyde TM, Caro JF. 1996 Autoradiographic localization of leptin binding in the choroid plexus of ob/ob and db/db mice. *Biochem Biophys Res Commun*. 219:884-889.
14. Considine RV, Considine EL, Williams CJ, Hyde TM, Caro JF. 1996 The hypothalamic leptin receptor in humans: identification of incidental sequence polymorphisms and absence of the db/db mouse and fa/fa rat mutations. *Diabetes*. 45:992-994.
15. Sinha MK, Opentanova I, Ohannesian JP, et al. 1996 Evidence of free and bound leptin in human circulation. *J Clin Invest*. 98:1277-1282.
16. Ahima RS, Prabakaran D, Mantzoros C, et al. 1996 Role of leptin in the neuroendocrine response to fasting. *Nature*. 382:250-252.
17. Chehab FF, Lim ME, Lu R. 1996 Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet*. 12:318-320.
18. Schwartz MW, Baskin DG, Bukowski TR, et al. 1996 Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. *Diabetes*. 45:531-535.
19. Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG. 1996 Identification of targets of leptin action in rat hypothalamus. *J Clin Invest*. 98:1101-1106.
20. Campfield LA, Smith FJ, Burn P. 1996 The OB protein (leptin) pathway - a link between adipose tissue mass and central neural networks. *Horm Metab Res*. 28:619-632.
21. Rohner-Jeanrenaud F, Cusin I, Sainsbury A, Zakrzewska KE, Jeanrenaud B. 1996 The loop system between neuropeptide Y and leptin in normal and obese rodents. *Horm Metab Res*. 28:642-648.
22. Chan YY, Steiner RA, Clifton DK. 1996 Regulation of hypothalamic neuropeptide-Y neurons by growth hormone in the rat. *Endocrinol*. 137:1319-1325.
23. Pierroz DD, Catzeflis C, Aebi AC, Rivier JE, Aubert ML. 1996 Chronic administration of neuropeptide Y into the lateral ventricle inhibits both the pituitary-testicular axis and growth hormone and insulin-like growth factor I secretion in intact adult male rats. *Endocrinol*. 137:3-12.
24. Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. 1995 Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med*. 1:1311-1314.
25. Maffei M, Halaas J, Ravussin E, et al. 1995 Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med*. 1:1155-1161.
26. Considine RV, Sinha MK, Heiman ML, et al. 1996 Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med*. 334:292-295.
27. Kolaczynski JW, Considine RV, Ohannesian J, et al. 1996 Responses of leptin to short-term fasting and refeeding in humans: A link with keto-genesis but not ketones themselves. *Diabetes*. 45:1511-1515.
28. Harris RB, Ramsay TG, Smith SR, Bruch RC. 1996 Early and late stimulation of ob mRNA expression in meal-fed and overfed rats. *J Clin Invest*. 97:2020-2026.
29. Kolaczynski JW, Ohannesian J, Considine RV, Marco C, Caro JF. 1996 Response of leptin to short-term and prolonged overfeeding in humans. *J Clin Endocrinol Metab*. 91:4162-4165.
30. Saladin R, De-Vos P, Guerre-Millo M, et al. 1995 Transient increase in obese gene expression after food intake or insulin administration. *Nature*. 377:527-529.

31. Cusin I, Sainsbury A, Doyle P, Rohner-Jeanrenaud F, Jeanrenaud B. 1995 The ob gene and insulin. A relationship leading to clues to the understanding of obesity. *Diabetes*. 44:1467-1470.
32. Kolaczynski JW, Nyce MR, Considine RV, et al. 1996 Acute and chronic effects of insulin on leptin production in humans: studies in vivo and in vitro. *Diabetes*. 45:699-701.
33. Malström R, Taskinen M-R, Karonen S-L, Yki-Järvinen H. 1996 Insulin increases plasma leptin concentrations in normal subjects and patients with NIDDM. *Diabetologia*. 39:993-996.
34. De-Vos P, Saladin R, Auwerx J, Staels B. 1995 Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. *J Biol Chem*. 270:15958-15961.
35. Sliker LJ, Sloop KW, Surface PL, et al. 1996 Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. *J Biol Chem*. 271:5301-5304.
36. Miell JP, Englaro P, Blum WF. 1996 Dexamethasone induces an acute and sustained rise in circulating leptin levels in normal human subjects. *Horm Metab Res*. 28:704-707.
37. Sinha MK, Ohannesian JP, Heiman ML, et al. 1996 Nocturnal rise of leptin in lean, obese, and non-insulin- dependent diabetes mellitus subjects. *J Clin Invest*. 97:1344-1347.
38. Havel PJ, Kasim-Karakas S, Dubuc GR, Mueller W, Phinney SD. 1996 Gender difference in plasma leptin concentrations. *Nature Med*. 2:949-950.
39. Rosenbaum M, Nicolson M, Hirsch J, et al. 1996 Effects of gender, body composition, and menopause on plasma concentrations of leptin. *J Clin Endocrinol Metab*. 81:3424-3427.
40. Hassink SG, Sheslow DV, de Lancey E, Opentanova I, Considine RV, Caro JF 1996 Serum leptin in children with obesity: relationship to gender and development. *Pediatrics*. 98:201-203.
41. Scholz GH, Englaro P, Thiele I, et al. 1996 Dissociation of serum leptin concentration and body fat content during long term dietary intervention in obese individuals. *Horm Metab Res*. 28:718-723.
42. Caro JF, Kolaczynski JW, Nyce MR, et al. 1996 Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. *Lancet*. 348:159-161.
43. Robinson CJ, Gaines-Das R, Woollacott D, et al. 2001 The first international standard for human leptin and the first international standard for mouse leptin: comparison of candidate preparations by in vitro bioassays and immunoassays. *J Molecular Endocrinol*. 27: 69-76.
44. Adresse NIBSC: Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.
45. Blum WF, Juul A; Reference ranges of serum leptin,s.318-326. In:Leptin -the voice of adipose tissue, Blum WF et al, eds., Johann Ambrosius Verlag, Heidelberg, 1997.

SUMMARY –hLEPTIN-SENSITIVE ELISA E077

| Reconstitution/ Dilution of reagents | | |
|---|--|--------|
| Standards A-E | reconstitute in Dilution Buffer VP (after using, store at –20°C) | 1 ml |
| Control Serum KS1&KS2 | reconstitute in Dilution Buffer VP (after using, store at –20°C) | 250 µl |
| Washing Buffer WP | dilute in A. dest. (e.g. add the complete contents of the flask (50 ml) into a graduated flask and fill with A.dest. to 1000 ml). | 1:20 |
| Sample dilution varies regarding the sample type, serum samples generally e.g. 1:10, use 100 µl per determination. | | |
| Before assay procedure bring all reagents to the room temperature . | | |

Proposal of Assay Procedure for double determinations

| Pipette | Reagents | Well positions |
|---------------------------------------|------------------------------------|---|
| 100 µl | Dilution Buffer VP as blank | A1 and A2 |
| 100 µl | Standard A (0.05 ng/ml) | B1 and B2 |
| 100 µl | Standard B (0.5 ng/ml) | C1 and C2 |
| 100 µl | Standard C (1.5 ng/ml) | D1 and D2 |
| 100 µl | Standard D (3.5 ng/ml) | E1 and E2 |
| 100 µl | Standard E (5 ng/ml) | F1 and F2 |
| 100 µl | Control Serum KS1 | G1 and G2 |
| 100 µl | Control Serum KS2 | H1 and H2 |
| 100 µl | Sample | Pipette sample in the rest of the wells according to requirements |
| Cover the wells with the sealing tape | | |

Incubation: 1 h at RT (20-25°C), 350 rpm

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

Incubation: 30 min at RT (20-25°C), 350 rpm

| | | |
|-----------|--|-----------|
| 3x 300 µl | Aspirate the contents of the wells and wash 3x with 300 µl Wash Buffer WP | each well |
| 100 µl | Substrate Solution S | each well |



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

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



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

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

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

 1 h **°C** 20-25  350 rpm

| | |
|-------------|----------------------|
| 3x 300 µl | 3x WASHBUF WP |
| 100 µl | AbCONJ AK |
| TAPE | |

 0.5 h **°C** 20-25  350 rpm

| | |
|-----------|---------------------------|
| 3x 300 µl | 3x WASHBUF WP |
| 100 µl | SUBST TMB S |

 15 min **°C** 20-25 

| | |
|----------------|---------------------------------------|
| 100 µl | H₂SO₄ SL |
| MEASURE | |