IGF-I ELISA

Enzyme Immunoassay for Quantitative Determination of

human Insulin-like Growth Factor I (IGF-I)

(IGFBP-blocked)

English

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

+2-8°C X 96 wells

h **E20**

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	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/ Conţinut suficient pentru x teste/ Vsebina zadostuje za x preizkusov/ Sisältö riittää x testille
Å	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
°C	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/ Pretrepať/ Protřepat/ Разклащане/ Raputada/ Ανακινήστε/ Vibrare/ Stresite/ Sekoita
MTP	Miktrotiterplate/ Mikrotiterplatte/ plaque de microtitrage/ Piastra di microtitolazione/ Placa de microtitulación/ Placa de Microtitulação/ Microtiterplaat/ Mikrotiterplade/ mikrotiterplatta/ microtiterplaat/ Płytka microtiter/ Mikrotiter lap/ Mikrotitračná podložka/ Mikrotitrační podložka/ Микротитърна плака/ Mikrotiiterplaat/ Τρυβλίο μικροτιτλοδότησης/ Microplacă/ Mikrotitrska plošča/ Mikrotitrauslevy
Rec in	Reconstitute in/ Rekonstituieren in/ Reconstituer dans/ Ricostituire nel/ Reconstituir en/ Reconstituir em/ Reconstitueren in/ Rekonstituér i/ Rekonstituera/ Rekonstytuować w/ Helyreállítás/ Znovu pripraviť za/ Znovu připravit za/ Разтваряне в/ Moodustada uuesti/ Ανασυστήστε σε/ Reconstituire în/ Predelava v/ Rekonstituoi
SPE	Sample/ Probe/ Echantillon/ Campione/ Muestra/ Amostra/ Monster/ Prøve/ prov/ Próbka/ Minta/ Vzorka/ Vzorek/ Проба/ Proov/ Δείγμα/ Probă/ Vzorec/ Näyte
DET	Antibody Conjugate/ Antikörperkonjugat/ Anticorps conjuguée/ Coniugato di anticorpo/ Conjugado de anticuerpos/ Conjugado anticorpo/ Antilichaamconjugaat/ Antistoffer-konjugat/ Antikroppskonjugat/ Koniugat antyciał/ Antitest páros/ Protilátkový konjugát/ Protilátkový konjugát/ Антитяло конюгат/ Antikehad konjugaat/ Σύμπλοκο αντισώματος*/* Compuşi din anticorpi/ Antitelesa konjugat/ Vasta-aine konjugaatti
EC	Enzyme Conjugate/ Enzymkonjugat/ Conjugué enzymatique/ Coniugato di enzima/ Conjugado de enzimas/ Conjugado Enzima/ Enzymconjugaat/ Enzym-konjugat/ Enzymkonjugat/ Koniugat enzymów/ Enzim páros/ Enzymatický konjugát/ Enzymatický konjugát/ ензим конюгат/ Ensüümi konjugaat/ Σύμπλοκο –ενζύμου/ Compuşi din enzime/ Encima konjugat/ Entsymi konjugaatti
SB	Sample Buffer/ Probenpuffer/ Tampon d'échantillon/ Buffer campione/ Tampón de muestra/ Tampão de amostra/ Monsterbuffer/ Prøvebuffer/ Provbuffert/ Bufor próbki/ Mintapuffer/ Pufr na vzorky/ Vzorkovací pufr/ Примерен буфер/ Proovipuhver/ Ρυθμιστικό διάλυμα δείγματος/ Tampon de probă/ Vzorčni puffer/ Näytepuskuri
X:X	Dilute / Verdünnen / Diluer / Diluire / Diluir / Diluir / Verdunnen / Fortyndes / Späd / Rozcieńczanie / Hígítás / Riediť / Ředit / Разреждане / Lahjendada / Αραιώστε / Diluaţi / Razredčiti / Laimennetaan

CAL	Calibrator X/ Kalibrator X/ calibrateur X/ calibratore X/ calibrador X/ calibrador X/ kalibrator X/ kalibrator X/ kalibrator X/ kalibrator X/ kalibrátor X/ kalibrátor X/ kalibrátor X/ калибратор X/ kalibraator X/ Βαθμονομητής X/ calibrator X/ kalibrator X/ kalibraattori X
CTR1 / CTR2	Control X/ Kontrolle X/ Contôle X/ controllo X/ control X/ Controle X/ controle X/ Kontrol X/ Kontroll X/ kontrolne X/ Ellenőrző X/ Kontrolné X/ Kontrolní X/ Контролен X/ Kontroll X/ ελέγχου X/ control X/ Kontrolni X/ Kontrolli X
WB	Washing Buffer Concentrate/ Waschpufferkonzentrat/ Tampon de lavage conc./ Tampone di lavaggio concentrato/ Tampón de lavado concentrado/ Tampão de Lavagem Concentrado/ wasbuffer, geconcentreerd/ Vaskebufferkoncentrat/ Vaskebufferkoncentrat/ tvättbuffertkoncentrat/ Bufor płukania koncentrat/ Mosópuffer koncentrátum/ Koncentrát vymývacieho pufra/ Концентрат на промивен буфер/ Pesupuhvri kontsentraat/ Συμπύκνωμα ρυθμιστικού διαλύματος έκπλυσης/ Concentrat pentru tamponul de spălare/ Koncentrat izpiralnega pufra/ Pesuliuositiiviste
WB 1:20	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/ Промивен буфер/ Pesupuhver/ Ρυθμιστικό διάλυμα έκπλυσης/ Tampon pentru spălare /Izpiralni pufer/ Pesuliuos
S	Substrate/ Substrat/ Substrat/ Substrato/ Substrato/ Substrato/ substraat/ Substrat/ Substrat/ Substrat/ Szubsztrátum/ Substrát/ Substrát/ Субстрат Substraat/ Υπόστρωμα/ Substrat/ Substrat/ Substraattiliuos
STP	Stop Solution/ Stopplösung/ Stop Solution/ Soluzione di stop/ Stop Solución/ Solução Stop/ stopoplossing/ Stopopløsning/ Stopplösning/ Stop roztwór/ Megállító oldat/ Roztok na ukončenie/ Roztok pro ukončení/ Стопиращ разтвор/ Stopp-lahus/ Διάλυμα διακοπής/ Soluţie 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 płytkę/ Tányér leragasztása/ Oblepiť podložku lepiacou páskou/ Olepit podložku lepicí páskou/ Плака с лента за запечатване/ Katta plaat isoleerkleeplindiga/ Κολλήστε το πλακίδιο με κολλητική ταινία/ Aoperiţi placa cu o bandă adezivă/ Prelepiti ploščo/ Peitä mikrotitrauslevy oheisella teipillä
MEASURE	Measure plate within 30 min at 450 nm (Referencefilter ≥590 nm)/ Ausmessung innerhalb von 30 min bei 450 nm (Referenzfilter ≥ 590 nm)/ Mesure lábsorbance en léspace de 30 min à 450 nm avec ≥590 nm longueur dónde 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 ≥ 590 nm)/ Binnen 30 minuten bij 450 nm meten (referentiefilter ≥ 590 nm)./ Mål plade i løbet af 30 min ved nm (referencefilter ≥590 nm)/ 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 (Referenčných filtrov ≥590 nm)/ Měřit 30 minut při 450 nm (Referenční filtr ≥ 590 nm)/ Отчитане в рамките на 30 min при 450 nm (референтен филтър ≥ 590 nm)/ Mõõtmine 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 referinţă ≥ 590 nm)/ Izmerite ploščico v 30 min pri 450 nm (referenčni filter ≥590nm)/ Mittaa 30 minuutin aikana 450 nm:ssä (referenssi suodatin ≥ 590 nm)
Literature	Literature/ Literatur/ Bibliographie/ Letterario/ Bibliografía/ Literatura documentação/ literatuur/ Litteratur/ litteratur/ 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/ instrucţiuni internaţionale 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 cavităţile necesare/ v vseh zahtevanih vdolbinah/ kaikkiin tarvittaviin mikrotitrauslevyn syvennyyksiin

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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.

IGF-I ELISA E20	**96 Determinations**
Principle of the test	Enzyme-linked Immunoassay
Duration (incubation period)	1.75 h
Antibodies	specific, monoclonal antibody and high-affinity polyclonal antiserum
Cross reactivity with IGF-II, Insulin, C-Peptide	< 0.1%
Buffers	Ready for use and 20fold concentrate
Calibrators	5 single Calibrators: 2 -50 ng/mL, recombinant human IGF-I
Reference material	International Standard WHO/NIBSC 02/254
Assay Range	0.09 – 1050 ng/mL
Controls	2 Controls, freeze-dried
Samples	human serum / plasma
Required sample volume	10 µL
Sample dilution	1:21
Analytical sensitivity	ø 0.09 µg/L
Intra- / Interassay Variance	ø < 10 %
Reference values	Blum W.F., Schweizer R. Insulin-Like Growth Factors and Their Binding Proteins. In: Ranke MB (ed): Diagnostics of Endocrine Function in Children and Adolescents. Basel, Karger, 2003, pp.166-199.

1 INTENDED USE

This enzyme immunoassay kit is for **research use only** and measures IGF-I in human serum or plasma or in various body fluids and in cell culture media.

2 INTRODUCTION

Insulin-like growth factors (IGF) I and II play a pivotal role in regulating the proliferation, differentiation and specific functions of many cell types (1-3). IGF-I is identical with Somatomedin C (Sm-C) (4) and has a molecular weight of 7649 Dalton (5). Its major regulators are growth hormone (GH) and nutrition (6), although its production in specific tissues is affected by a multitude of tropic hormones and other peptide growth factors. IGFs are avidly bound to specific binding proteins (IGFBP). The seven classes of IGFBPs which are known at present (7,8,22). A major problem of IGF-I measurement results from the interference of IGFBPs in the assay. Direct determinations in untreated serum samples (11) give false values because of the extremely slow dissociation of the IGF-I/IGFBP-3 complexes during the assay incubation.

IGF-I is present in low concentrations in various body fluids and in conditioned cell culture media of many cell lines. However, the determination of IGF-I in these specimens is particularly difficult due to the presence of IGFBPs usually in excessive amounts. The IGFBP-blocked IGF-I ELISA avoids these problems and allows the simple, correct and sensitive IGF-I determination in numerous samples with minimal expenditure of time.

Therefore, various techniques were applied to physically separate IGF-I from its binding proteins before measurement, including (a) size exclusion chromatography under acidic conditions, (b) solid-phase extraction and (c) acid-ethanol extraction (2,12,13). These

techniques, however, are either inconvenient or time-consuming or give incomplete and not-reproducible recoveries. The most widely used method is the acid-ethanol extraction (13,14) with a recovery of only 70-80 % of IGFBP-bound IGF-I as a result of co-precipitation. The absolute results of such an extraction are therefore false low (15). The extraction removes the IGFBPs only insufficiently and leads to reduction in sensitivity of the assay due to pre-dilution of the samples by the extraction procedure. Furthermore, the remaining IGFBP may still interfere in the assay. In addition, the acid-ethanol extraction is ineffective in specimens other than serum or plasma (e.g. cell culture media), in which determination of IGF-I is already difficult enough due to the fact that IGFBPs are frequently present at large excess.
To avoid these difficulties, an uncomplicated assay was developed, in which special sample preparation is not required before measurement.

3 PRINCIPLE

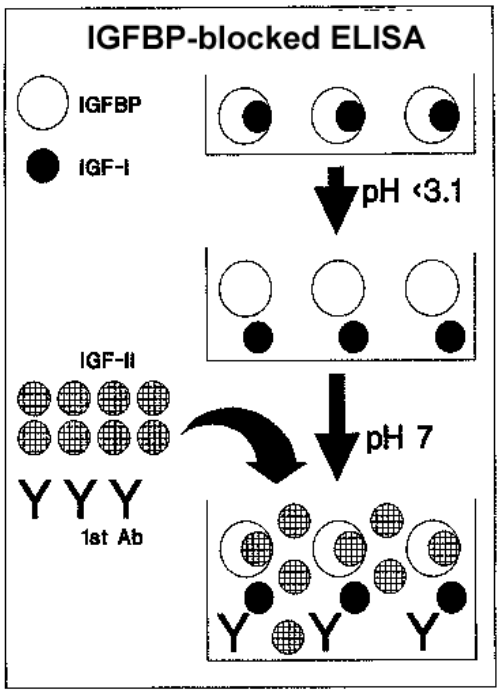

Figure 2 Principle of the IGFBP-blocked IGF-I ELISA

In order to dissociate IGF-I from the IGFBPs, the samples must be diluted in an acidic buffer (**Sample Buffer SB)** (Figure 2). The diluted samples are then pipetted into the assay wells. The IGF-I antiserum is dissolved in a buffer, which is able to neutralize the acidic samples. After the IGF-I antibody solution has neutralized the samples, the present excess IGF-II occupies the IGF-binding sites of the binding proteins, thus allowing the measurement of the resulting free IGF-I. With this method, the IGFBPs are not removed, but their function and therefore their interference in the assay is neutralized. Due to the extremely low cross-reactivity of the IGF-I antibody with IGF-II, the excess of IGF-II does not disturb the interaction of the first antibody with IGF-I.
The Mediagnost ELISA for IGF-I E20 is a so-called Sandwich-Assay using two specific and high affinity antibodies. The IGF-I in the samples binds to the first antibody coated on the microtiterplate, the second specific anti-IGF-I-antibody binds in turn to the immobilised IGF-I. The second antibody is biotinylated, the subsequently incubated Streptavidin-Peroxidase-Enzyme Conjugate will bind to it, and thus in the final substrate incubation step colour development will be catalysed quantitatively depending on the IGF-I-level of the samples. The Calibrators of the ELISA E20 are prepared from recombinant IGF-I in concentrations of 2, 5, 15, 30 and 50 ng/mL.

4 WARNINGS AND PRECAUTIONS

For in-vitro use only. For Professional use only.
For Research Use Only. For Professional use only.

The Mediagnost kit is suitable only for in vitro and research use and not for internal use in humans or 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.

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

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

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 Controls 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 specimens should be treated as potentially infectious.

Reagents CAL A-E, DET, EC, SB, 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_2SO_4)

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

4.1 General first aid procedures:

Skin contact: Wash affected area rinse immediately with plenty of water at least 15 minutes. Remove contaminated cloths and shoes.
Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing, spread the eyelids.
Ingestion: After swallowing the product, if the affected person is conscious, rinse out the mouth with plenty of water: seek medical advice immediately.

5 SAMPLES

5.1 Sample type

Serum and Plasma
Serum and Heparin/EDTA Plasma yield comparable values. The IGF-I levels are reduced in citrate plasma samples, because of the relatively high amount of anticoagulant.

Furthermore, suitable samples are: cerebrospinal and urine samples as well as cell culture medium. IGF-I levels can vary considerably, the optimal dilution must be found out by the user.

5.2 Specimen collection

Use standard venipuncture for the blood sampling. Hemolytic reactions are to be avoided.

5.3 Required sample volume: 10 µL

5.4 Sample stability

In firmly closed sample vials:

- Storage at 20-25°C max. 24 hours
- Storage at -20°C : min. 2 years
- Freeze-thaw cycles max. 3

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. 3 freeze-thaw cycles showed no effect on samples.

5.5 Interference

Triglyceride, bilirubin and hemoglobin in the sample do not interfere to a concentration of 100 mg/mL and 200 µg/mL or 1 mg/mL. However, the use of hemolytic, lipemic or icteric samples should be validated by the user.

5.6 Sample dilution

- Dilution: **1:21** with **Sample Buffer SB**
- Pipette **200 µL Sample Buffer SB** in PE-/PP-Tube (application of a multi-stepper is recommended in larger series); add **10 µL sample** (dilution 1:21). After mixing use 2 x 20 µL of this dilution in the assay.
- Attention: serum and plasma samples must be diluted at least 1:10 in **Sample Buffer SB** in order to achieve sufficient acidification of the samples.
- Depending on the expected IGF-I values the samples can be diluted higher in **Sample Buffer SB**.
- Sample stability after dilution of the sample: maximum 2 hours at 20-25°C.

6 MATERIALS

6.1 Materials provided

The reagents listed below are sufficient for 96 wells including the Calibration Curve.

MTP	**Microtiter plate,** ready for use, coated with mouse-anti-hIGF-I-antibody. Wells are separately breakable.	**(8x12) wells**
CAL A-E	**Calibrators**, lyophilized, (recombinant human hIGF-I), concentrations are given on vial labels and on quality certificate.	**5 x 500 µL**
CTR1	**Control 1,** lyophilised, (human serum), concentration is given on quality certificate.	**1 x 500 µL**
CTR2	**Control 2,** lyophilised, (human serum), concentration is given on quality certificate.	**1 x 500 µL**
DET	**Antibody Conjugate,** ready for use, contains goat biotinylated anti-hIGF-I antibody**.**	**1 x 9 mL**
EC	**Enzyme Conjugate,** ready for use, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin.	**1 x 12 mL**
SB	**Sample Buffer,** ready for use.	**1 x 25 mL**
WB	**Washing Buffer,** 20-fold concentrated solution	**1 x 50 mL**
S	**Substrate,** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised Tetramethylbencidine.	**1 x 12 mL**
STP	**Stop Solution,** ready for use, 0.2 M sulphuric acid.	**1 x 12 mL**
-	**Sealing Tape,** for covering the **microtiter plate.**	**2 x**
	**Instructions for use**	**1 x**
-	**Quality Certificate**	**1 x**

6.2 Materials required, but not provided

- Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer **WB (A. dest.), 950 mL.**
- Precision pipettes and multichannel pipettes with disposable plastic tips
- Polyethylene PE/Polypropylene PP tubes for dilution of samples
- Vortex-mixer
- Microtiter plate shaker (350 rpm)
- Microtiter plate washer (recommended)
- Micro plate reader ("ELISA-Reader") with filter for 450 and [3] 590 nm

7 TECHNICAL NOTES

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

Storage Life
The shelf life of the components **after initial opening** is warranted for **4 weeks**, store the unused strips and microtiter wells **airtight** together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The **reconstituted components** 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.
Do not use expired reagents. Temperature WILL affect the absorbance readings of the assay. However, correctness of the results 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 microtiterplate wells thoroughly to ensure good test results. Do not reuse microtiterplate wells.

Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.

Reconstitution
The Calibrators **A–E** and Control **CTR1 and CTR2** are reconstituted with the Sample Buffer **SB**. 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
After reconstitution dilute the Control **CTR1** and **CTR2** with the Sample Buffer **SB** in the same ratio (1:21) as the sample.
The required volume of Washing Buffer **WB** is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest..

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

Shaking
The incubation steps should be performed at mean rotation frequency of a microtiter plate shaker. We recommend 350 rpm. Depending on the design of the shaker, the shaking frequency should 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 **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.

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

NOTES: When performing the assay, Blank, Calibrators **A-E**, Controls **CTR1 and CTR2** and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody Conjugate **DET,** Enzyme Conjugate **EC** and 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**, Control **CTR1** and **CTR2** and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

1) Add **80 µL Antibody Conjugate DET** in **all** wells used.
2) Pipette in positions A1/2 **20 µL Sample Buffer SB.**
3) Pipette in positions B1/2 **20 µL of the Calibrator A (2 ng/ml)**
   Pipette in positions C1/2 **20 µL of the Calibrator B (5 ng/ml)**,
   Pipette in positions D1/2 **20 µL of the Calibrator C (15 ng/ml)**,
   Pipette in positions E1/2 **20 µL of the Calibrator D (30 ng/ml)**,
   Pipette in positions F1/2 **20 µL of the Calibrator E (50 ng/ml)**.
   To control the correct accomplishment of the assay **20 µL** of the 1:21 (or in respective dilution ratio of the samples) in **Sample Buffer SB** diluted **Controls CTR1&CTR2** can be pipetted in positions G1/2 and H1/2.
   Pipette **20 µL** each of the diluted sample (e.g. dilute 1:21 with Sample Buffer **SB**) in the rest of wells, according to your requirements.
4) Cover the wells with sealing tape and incubate the plate for **1 hour** at **room temperature** 20-25°C (shake at 350 rpm).
5) After incubation aspirate the contents of the wells and wash the wells 5 times **300 µL Washing Buffer WB** / well.
6) Following the last washing step pipette **100 µL** of the **Enzyme Conjugate EC** in each well.
7) Cover the wells with sealing tape and incubate the plate for **30 minutes** at **room temperature** 20-25°C (shake at 350 rpm).
8) After incubation wash the wells 5 times with **Washing Buffer WB** as described in step 5.
9) Pipette **100 µL** of the **Substrate S**.
10) Incubate the plate for **15 minutes in the dark** at **room temperature** 20-25°C
11) Stop the reaction by adding **100 µL Stop Solution STP** to all wells.
12) Measure the absorbance within **30 minutes** at **450 nm**
    (**Reference filter** [3] **590 nm**).

9 EVALUATION OF RESULTS

9.1 Establishing of the Calibration Curve

The International Standard for hIGF-I, WHO NIBSC Code 02/254 was used as calibrator material and the following IGF-I concentrations are used. 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.

Calibrator	A	B	C	D	E
ng/mL	2	5	15	30	50
nmol/L	0.26	0.66	1.96	3.92	6.54

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 IGF-I concentration in ng/mL of the samples and controls can be calculated by **multiplication** with the respective **dilution factor.**

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	2	5	15	30	50
OD(450-620 nm)	0.00	0.088	0.299	0.985	1.727	2.543

The exemplary shown Calibration Curve in **Figure 3 cannot** be used for calculation of your test results. You have to establish a Calibration Curve for each test you conduct!

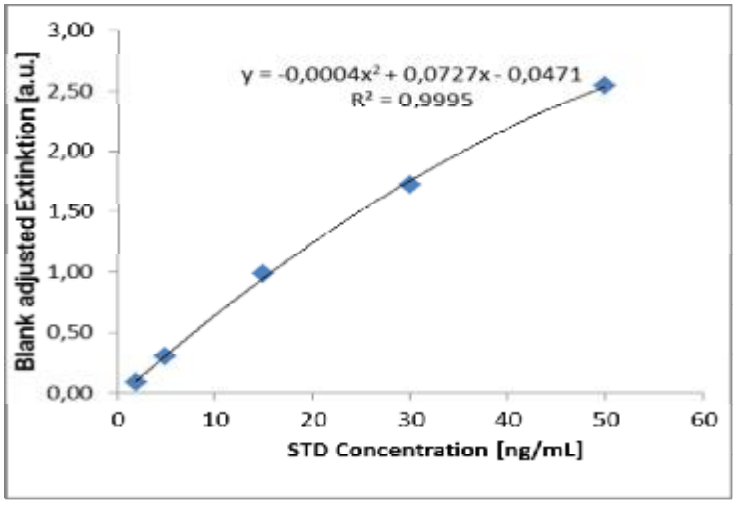

Figure 3 Examplary Calibration Curve

9.3 Exemplary calculation of IGF-I concentrations

Sample dilution: 1:21

Measured extinction of the blank	0.0165
Measured extinction of your sample	0.2695

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

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

$0.253 = -0.0004x^2 + 0.0727x - 0.0471$

$4.57 = x$

If the dilution factor (**1:21**) is taken into account the **IGF-I** concentration of the undiluted sample is

4.57 ng/mL ´ 21 = 96 ng/mL

9.4 Interpretation of results

The test is for research use only. Not for use in diagnostic procedures. Therefore, interpretation of test results depends on experimental settings and scientific question.

10 LIMITATIONS OF PROCEDURE

The Mediagnost IGF-I ELISA, E20 is based on a combination of monoclonal capture and polyclonal detection antibodies. Generally, immunological assays are sensible to heterophilic antibodies and rheumatoid factors in the sample. Their influence is reduced by the assay design, but cannot be excluded completely.

11 EXEMPLARY VALUES

IGF-I levels are highly age-dependent in children, less so in adults until the age of about 60. The exemplary ranges in various age groups, which are log-normally distributed, are given in Table 2 by percentiles.
Between 8 and 19 years of age, values are given for boys and girls separately, because the pubertal peak usually occurs approximately 2 years earlier in girls.

Table 1 Exemplary range of serum IGF-I levels given in ng/mL at different pubertal stages according to Tanner. Because no significant difference between boys and girls is observed, both sexes are combined. Only children and adolescents between 7 and 17 years of age are included.

	Percentile			
Pubertal Stage	**0.1th**	**5th**	**50th**	**95th**
1	61	105	186	330
2	85	156	298	568
3	113	196	352	631
4	171	268	431	693
5	165	263	431	706

Table 2 Serum levels of IGF-I in healthy subjects at various ages. Individuals between 8 and 19 years of age were classified according to gender, as the pubertal peak occurs almost 2 years earlier in girls than in boys.

	Percentile													
Age	**0.1**	1	**5**	10	**20**	30	**40**	50	**60**	70	**80**	90	**95**	99
0-2 y.	**13**	20	**28**	34	**43**	50	**58**	66	**75**	87	**102**	128	**156**	220
2-4 y.	**20**	29	**40**	48	**59**	68	**77**	87	**98**	111	**129**	159	**189**	260
4-6 y.	**26**	36	**50**	59	**73**	85	**96**	108	**122**	138	**160**	196	**233**	320
6-7 y.	**34**	46	**62**	72	**87**	99	**111**	124	**138**	155	**176**	212	**248**	332
7-8 y.	**45**	60	**78**	90	**107**	121	**134**	148	**163**	181	**205**	243	**281**	364
8-9 y. boys	**54**	71	**90**	102	**119**	133	**146**	160	**175**	192	**214**	250	**284**	362
girls	**55**	75	**99**	115	**137**	156	**174**	193	**214**	239	**271**	324	**376**	496
9-10 y. boys	**63**	82	**102**	115	**133**	148	**162**	176	**191**	209	**232**	269	**304**	379
girls	**68**	89	**114**	130	**152**	170	**187**	205	**224**	247	**276**	323	**369**	469
10-11 y. boys	**77**	96	**117**	130	**148**	162	**176**	189	**203**	220	**241**	274	**305**	370
girls	**81**	106	**134**	153	**178**	199	**219**	239	**261**	287	**321**	374	**426**	539
11-12 y. boys	**85**	106	**129**	144	**163**	179	**194**	209	**225**	244	**267**	304	**339**	413
girls	**91**	123	**160**	185	**220**	248	**276**	305	**337**	374	**424**	503	**581**	758
12-13 y. boys	**88**	112	**141**	159	**184**	204	**223**	243	**264**	289	**321**	371	**419**	525
girls	**116**	155	**201**	231	**274**	309	**342**	377	**415**	460	**519**	614	**707**	914
13-14 y. boys	**111**	143	**179**	203	**235**	261	**286**	311	**339**	371	**412**	477	**540**	677
girls	**163**	207	**256**	287	**329**	364	**395**	428	**463**	504	**556**	637	**716**	884
14-15 y. boys	**140**	182	**229**	260	**303**	337	**370**	404	**441**	484	**539**	625	**691**	896
girls	**193**	236	**284**	314	**353**	385	**414**	443	**474**	510	**556**	628	**713**	832
15-16 y. boys	**176**	221	**269**	299	**340**	372	**402**	433	**466**	504	**552**	626	**697**	849
girls	**187**	231	**279**	309	**350**	382	**412**	442	**474**	512	**559**	632	**700**	845
16-17 y. boys	**178**	221	**267**	296	**335**	366	**395**	424	**455**	491	**537**	607	**673**	814
girls	**183**	225	**270**	298	**336**	366	**394**	422	**452**	486	**530**	597	**660**	792
17-18 y. boys	**173**	207	**243**	265	**294**	317	**337**	358	**380**	405	**436**	484	**527**	618
girls	**176**	210	**246**	268	**297**	320	**341**	362	**384**	409	**441**	488	**533**	624
18-19 y. boys	**167**	201	**235**	256	**285**	307	**327**	347	**368**	393	**423**	469	**512**	600
girls	**167**	199	**233**	254	**281**	302	**322**	341	**362**	385	**414**	458	**499**	583
19-20 y.	**158**	189	**220**	240	**265**	285	**304**	322	**341**	363	**391**	433	**471**	550
20-30 y.	**72**	92	**115**	130	**150**	167	**182**	198	**215**	235	**261**	302	**340**	425
30-40 y.	**68**	87	**109**	123	**142**	158	**173**	188	**204**	223	**248**	287	**324**	404
40-50 y.	**64**	82	**103**	116	**135**	150	**164**	178	**194**	212	**235**	272	**310**	385
50-60 y.	**60**	77	**97**	110	**127**	142	**155**	169	**184**	201	**224**	260	**292**	369
60-70 y.	**55**	72	**91**	103	**120**	134	**147**	161	**176**	193	**215**	251	**282**	362
70-80 y.	**25**	35	**47**	55	**67**	78	**88**	98	**110**	124	**142**	173	**207**	276
>80 y.	**21**	30	**40**	47	**58**	67	**76**	85	**95**	108	**125**	153	**184**	245

Serum concentrations are given in ng/ml.

Exemplary values have been evaluated by Prof Blum by a radioimmunoassay identical to Mediagnost IGF-R20. Thus, the age and sex specific exemplary values published in Diagnostics of Endocrine Function in Children and Adolescents.
(Edited by Prof Ranke. ISBN-3-335-00496-5) can be applied to all Mediagnost IGF-I assays.

12 PERFORMANCE CHARACTERISTICS

12.1 Sensitivity

Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the blank + 2SD. The analytical sensitivity of the Mediagnost E20 is 0.091 ng/mL on average, in 19 independent determinations values from 0.03 ng/mL to 0.2 ng/mL were found.

12.2 Specificity

The measurements of E20 cross reactivity with IGF-II, Insulin and C-Peptide. These IGF-related proteins were added to assay buffer in the indicated concentration and the solution was applied as sample without any further dilution. The concentration measured within the blank without any protein was 0.78 µg/L. Thus, neither IGF-II nor Insulin or C-Peptide are measured by the Mediagnost E20 ELISA (see table 3).

Table 3 Specificity. Cross reactivity of the test system with different IGF-I related proteins.

added C-Peptide [µg/L]	measured IGF-I [µg/L]	added Insulin [µg/L]	measured IGF-I [µg/L]	added IGF-II [µg/L]	measured IGF-I [µg/L]
500	0.73	100	0.78	1250	0.77
100	0.78	10	0.77	750	0.73
10	0.77	1	0.76	250	0.77
0	0.78	0	0.78	0	0.78

12.3 Cross Reactions with Animal Samples

Several commercially available animal sera have been used as samples in this assay and therewith it is proven, that the test can be used as heterologous assay for IGF-I measurement in serum samples of primates, cattle, pig, sheep, horse, donkey, goat, dog, cat, rabbit and guinea pig. For the determination of IGF-I in chicken, rat and mouse sera is this kit not usable. Species specific calibration has to be done by the user.
To determine exact species-specific IGF-I concentrations, the test system must be calibrated respectively: for example, using bovine IGF-I in a known concentration.

12.4 Precision

Intra-Assay Variance

Three samples have been measured severalfold in the same assay. The results are shown in table 4. The measured coefficient of variation (CV) is 5.81% on average.

Table 4 Intra-Assay variability

	Number of determinations	Mean value (ng/mL)	Standard deviation (ng/mL)	VC (%)
Sample 1	18	144.8	9.63	6.65
Sample 2	18	140.79	7.15	5.08
Sample 3	18	138.02	7.86	5.69

Inter-Assay and Lot-to-Lot Variance

Serum samples where measured in independent assays. Exemplary results are shown in Table 5. Further, five samples were also tested repeatedly throughout four years in eight different lots. The variability on average is 8.57% (6.8 – 10.5%).

Table 5 Inter-Assay variability

	Number of determinations	Mean value [ng/mL]	Standard deviation [ng/mL]	VC [%]
Sample 1	8	81	5.34	6.56
Sample 2	16	192	12.38	6.43
Sample 3	17	498	27.52	5.53

12.5 Linearity

Linearity was tested by dilution of native sera with different IGF-I contents (Sample 1-3). The amount of measured IGF-I was recalculated and is shown in Figure 4.

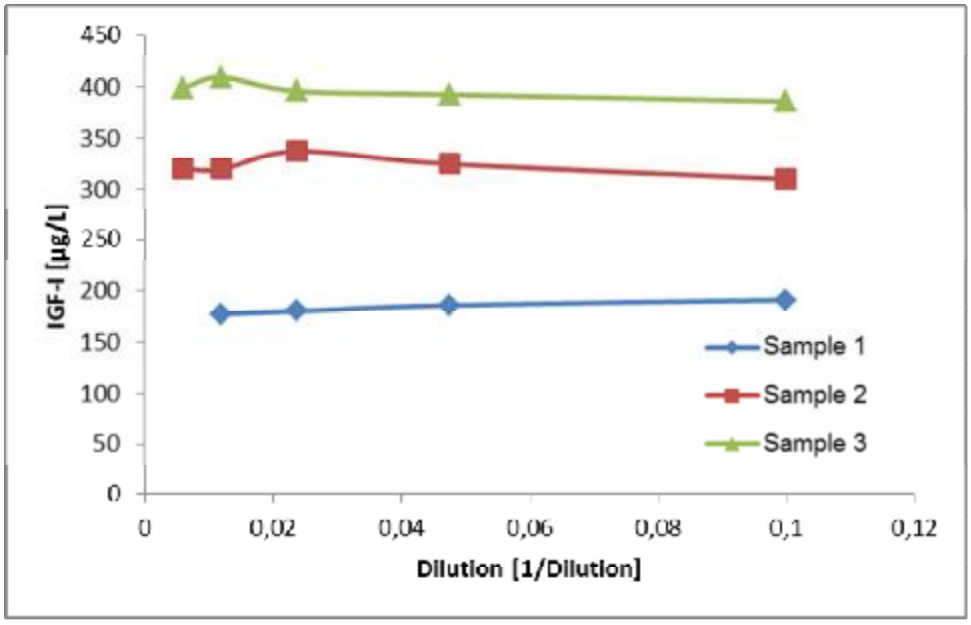

Figure 4 Linearity, recalculated IGF-I concentrations of differentially diluted samples. The minimal dilution is 1:10, the recommended dilution is 1:21.

12.6 Recovery and Accuracy

Recombinant IGF-I was added in different amounts to human serum. The IGF-I content of the so enriched samples was measured and recovery in comparison to enriched buffer calculated. Results are shown in Table 6.

Table 6 Recovery of recombinant IGF-I in human serum

IGF-I [µg/L]	Sample 1	Sample 2	Sample 3	Sample 4
Sample	138	172	133	180
+ IGF-I 200	287	372	-	-
+ IGF-I 400	-	-	539	591
% Recovery	**85**	**100**	**101**	**102**

12.7 Interference

Interference of bilirubin and triglycerides was tested by adding different amounts of these substances to human serum containing IGF-I. For comparison the same amount of buffer without any substance was also added to the serum. Table 7 demonstrates that neither bilirubin nor triglycerides exert any influence on the measurement of IGF-I in human serum.

Table 7 Interference of physiologic substances on IGF-I measurement. Human serum samples were enriched with different amounts of trigylcerides, bilirubin or hemoglobin and the recovery of IGF-I was measured. Here the relative recovery in [%] of not enriched samples is shown.

	Triglyceride 100 mg/mL	Bilirubin 200 µg/mL	Hemoglobin 10 mg/ml
Serum 1	93	90	97
Serum 2	100	101	110
Serum 3	120	120	104

Influence of binding proteins on IGF-I measurement was exemplarily elucidated with IGFBP-3.

Different amounts of IGF-I and 3 or 6 mg/L of IGFBP-3 were added to sample buffer (pH 2) and phosphate based saline buffer (pH 7.4). After a short incubation of 15 minutes at room temperature these samples were diluted and applied to the Mediagnost E20 as described in

the package insert. In case of sample buffer IGFBP-3 up to 6 mg/L did not interfere with IGF-I measurement. But without acidification of the sample a strong interference of IGFBP-3 with IGF-I measurement was detected (Table 8).

Table 8 Interference of IGFBP-3 with IGF-I measurement

IGFBP-3	Sample Buffer		
	50 µg/L IGF-I	100 µg/L IGF-I	300 µg/L IGF-I
-	46.38	116.14	358.1
3 mg/L	47.33	115.83	384.15
6 mg/L	52.32	123.38	355.41
IGFBP-3	**Phosphate buffered Saline**		
	50 µg/L IGF-I	**100 µg/L IGF-I**	**300 µg/L IGF-I**
-	34.2	90.23	349.04
3 mg/L	7.4	12.16	152.14
6 mg/L	7.2	10.12	48.15

12.8 Assay Calibration

Recombinant human IGF-I produced by E. coli and of >98% purity (SDS-PAGE, Silverstain) is used as Calibrator within the assay. This recombinant hIGF-I devoid of methIGF-I or IGF-I variants with mismatched disulfide bonds is identical to the major authentic IGF-I form in blood. The traceability of this recombinant Calibrator material to the international reference material of the WHO 02/254 has been proven. Results are published by Burns C et al. in Growth Horm IGF Res. 2009 Oct;19(5):457-62. Epub 2009 Mar 20. Mediagnost E20 ELISA is coded by 14c.

The reference material includes **8.5 µg/ampoule** IGF-I measured by amino acid analysis and HPLC. Mediagnost E20 immunoassay (assay No. 14c) measures **11.55 µg/ampoule**. The mean of all tested immunoassays is 11.61 µg/ampoule.

Thus, Mediagnost results are comparable to other immunological tests for measurement of IGF-I and can easily be transformed to WHO 02/254 (25, 26) by a factor of **0.735**.

13 ASSAY COMPARISON

Mediagnost E20 IGF-I was compared with the Mediagnost R20 IGF-I. 196 serum samples were measured in both assays and an excellent coefficient of correlation was shown with r = 0.95. Additionally, the Mediagnost IGF-I ELISA E20 was compared with an Enzyme-Immunoassay of other commercially available IGF-I test and a correlation of R^2>0.9 was shown.

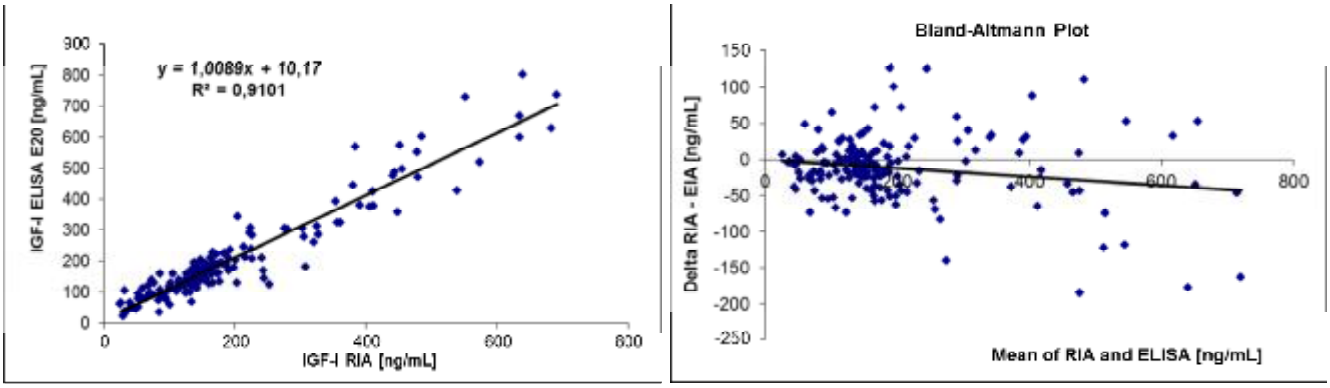

Figure 5 Assay Comparison Mediagnost RIA R20 and Mediagnost ELISA E20

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15 INTERNATIONAL ASSAY DESCRIPTION

International Test Description

CAL A-E	**Rec in 500 µL SB**	**-**
CTR1	**Rec in 500 µL SB**	**1:21 SB**
CTR2	**Rec in 500 µL SB**	**1:21 SB**
WB 20x	**-**	**1:20 A. dest. è WB 1:20**
SPE		**1:21 SB**
-	**°C 20-25°C, À ↔ max. 2 h**	

80 µL	**DET**	A1 - End
20 µL	**SB**	A1/A2
20 µL	**CAL A (2 ng/ml)**	B1/B2
20 µL	**CAL B (5 ng/ml)**	C1/C2
20 µL	**CAL C (15 ng/ml)**	D1/D2
20 µL	**CAL D (30 ng/ml)**	E1/E2
20 µL	**CAL E (50 ng/ml)**	F1/F2
20 µL	**CTR1 1:21 SB**	G1/G2
20 µL	**CTR2 1:21 SB**	H1/H2
20 µL	**SPE 1:21 SB**	
TAPE		
À 1 h °C 20-25°C ↔ 350 rpm		
5x 300 µL	**5x WB 1:20**	
100 µL	**EC**	
TAPE		
À 0.5 h °C 20-25°C ↔ 350 rpm		
5x 300 µL	**5x WB 1:20**	
100 µL	**S**	
À 15 min °C 20-25°C		
STP		
MEASURE		

16 ASSAY PROCEDURE

Preparation of reagents		**Reconstitution:**	**Dilution**
CAL A-E	**Calibrators**	in **500 µL** Sample Buffer **SB**	-
CTR1	**Control 1**	in **500 µL** Sample Buffer **SB**	**1:21** with Sample Buffer **SB**
CTR2	**Control 2**	in **500 µL** Sample Buffer **SB**	**1:21** with Sample Buffer **SB**
WB	**Washing Buffer conc.**		**1:20** with **Aqua dest.** **è WB 1:20**
Samples + Controls CTR1 and **CTR2**: dilute 1:21 in Sample Buffer **SB**, mix immediately, incubate max. 2h. Use **20 µL** for each well in the assay.			
Before assay procedure bring all reagents to room temperature **(20-25°C)**.			

Assay procedure in double determination		
Pipette	**Reagents**	**Position**
80 µL	Antibody Conjugate **DET**	in **all** wells used
20 µL	Sample Buffer **SB** (Blank)	A1/A2
20 µL	Calibrator **A** (**2 ng/mL**)	B1/B2
20 µL	Calibrator **B** (**5 ng/mL**)	C1/C2
20 µL	Calibrator **C** (**15 ng/mL**)	D1/D2
20 µL	Calibrator **D** (**30 ng/mL**)	E1/E2
20 µL	Calibrator **E** (**50 ng/mL**)	F1/F2
20 µL	Control **CTR1** (1:21 diluted)	G1/G2
20 µL	Control **CTR2** (1:21 diluted)	H1/H2
20 µL	Sample **SPE** (1:21 diluted)	in the rest of the wells according the requirements
Cover the wells with the sealing tape.		
Sample Incubation: 1 h at 20-25°C, 350 rpm		
5x 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	Enzyme Conjugate **EC**	In each well
Cover the wells with the sealing tape.		
Incubation: 30 Minutes at 20-25°C, 350 rpm		
5x 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** with ≥ 590 nm as reference wavelength.		