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IGF-II ELISA

Enzyme Immunoassay for Quantitative Determination of

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

(IGFBP-blocked)
English

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

+2-8°C

96 wells

REF **E30**

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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
	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/ 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 cover foil/ 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.

ENGLISH - Instructions for use

IGF-II ELISA	96 Determinations
Principle of the test	Enzyme Immunoassay
Duration (incubation period)	3 h
Antibody Conjugate	ready for use
Enzyme Conjugate	ready for use
Buffer	ready for use and 20fold concentrate
Substrate	ready for use
Calibrators	5 single calibrators: 0.45 - 9 ng/mL, lyophilized, human recombinant IGF-II
Reference material	Calibrated against the International Standard **WHO/NIBSC 96/538**
Assay Range	0.06 – 3636 ng/mL
Control	2 controls, lyophilised
Sample	human serum / plasma
Required sample volume	10 µL
Sample dilution	1:404
Analytical sensitivity	Ø 0.06 ng/mL
average Intra- / Inter-Assay Variance	Ø < 10%
Examplary Values	Blum W.F et.al.1990 Insulin-Like Growth Factors and Their Binding Proteins. In: Ranke MB, Mullins P.E. (ed): Diagnostics of Endocrine Function in Children and Adolescents. Basel, Karger, 2011, pp.157-181

1 INTENDED USE

Measurement of IGF-II in human serum, plasma, urine, saliva as well as cell culture supernatants for research use only!

2 INTRODUCTION

The insulin-like growth factors (IGF)-I and –II play a pivotal role in the regulation of proliferation and differentiation of several tissue types (1-3). IGF-I also called Somatomedin C (4) has a molecular weight of 7.469 kDa (5). Its expression is mainly regulated by Growth Hormone and nutrition (6). But several hormones and peptide factors are known to influence IGF-II synthesis in different tissues. Bioavailability of the IGFs is regulated by specific binding proteins (IGFBP). Beside the high affinity Insulin-like Growth Factor Binding Proteins 1-6, IGFs are also bound be IGFBP-related Proteins (7, 8, 22). These binding proteins bind IGF-I and IGF-II with the same affinity or prefer IGF-II (9, 10). Direct measurement of IGFs in serum samples without pretreatment results in false values because of the extremely slow dissociation of the IGF/IGFBP complexes during the assay incubation only a part of the IGF-II in the specimen can bind to the antibodies and be detected.

Therefore, various techniques were applied to physically separate IGF-II 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.

This assay is easy, fast and results do not depend on the binding protein concentration of the sample. It is based on the high specificity of the employed antibodies for IGF-II. There is virtually no cross-reactivity with IGF-I. This allows the separation of IGF-II from the binding proteins by acidification and blocking of the free binding proteins with IGF-I. Thus, the endogenous IGF-II is free in solution.

3 ASSAY PRINCIPLE

The Mediagnost ELISA for IGF-II E30 is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The first antibody, immobilized on the microtiter plate, and the added second biotinylated antibody are binding the IGF-II in the sample. The Streptavidin-Peroxidase Enzyme Conjugate subsequently binds to the complex. In the closing substrate reaction, the turn of the colour will be high specific catalysed, quantitatively depending on the IGF-II-level of the samples.

IGF-II-IGFBP complex is dissociated by dilution in an acidic buffer. IGFBPs are blocked by IGF-I excess, thus allowing the measurement of free IGF-II. With this method, the IGFBPs are not removed, but their function and therefore their interference in the assay is neutralized.

Due to the low cross-reactivity of the IGF-II antibody with IGF-I, excess IGF-I does not disturb the interaction of the first antibody with IGF-II

4 WARNINGS AND PRECAUTIONS

For in-vitro use only. For Professional use only. For Research Use Only. Not for use in diagnostic procedures. Not for internal use in humans and animals. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Mediagnost will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.

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

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

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

Human Serum
Following components contain human serum: **Controls CTR1 / 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.

4.1 Components

All components of the product that contain hazardous substances in declarable concentrations in accordance with European Regulation 1272/2008 are listed below:

Antibody Conjugate DET / Enzyme Conjugate EC
The buffers contain the hazardous ingredients listed below, based on this the following labeling of the two mixtures results:

Hazard pictograms (CLP):

GHS07

Signal word (CLP):	Warning
Hazardous components:	2-methylisothiazol-3(2H)-one (< 0,06 %) reaction mass of 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (3:1) (< 0,015 %)
Hazard statements (CLP):	H317 - May cause an allergic skin reaction.
Safety instructions (CLP):	P280 - Wear protective gloves/protective clothing/eye protection/face protection/hearingprotection. 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 Water. P501 - Dispose of contents/container in accordance with national disposal regulations.

Washing Buffer WB / Sample Buffer SB
The buffers contain a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (< 0.015 %) as a preservative.

EUH208	Contains reaction mass of 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (3:1). May produce an allergic reaction.
EUH210	Safety data sheet available on request.

Stop Solution STP
The stop solution contains 0.2 M sulphuric acid.

EUH210	Safety data sheet available on request.

4.2 Protective equipment and first aid measures

After contact with skin:	Take off immediately all contaminated clothing. Rinse skin with water or shower. In case of skin reactions, consult a physician.
After contact with eyes:	Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. In case of eye irritation consult an ophthalmologist.
After ingestion:	Call a doctor if you feel unwell.

Avoid contact with the ingredients. Detailed information on personal protective equipment and first aid measures can be found in the safety data sheet for IGF-II ELISA E30. This can be accessed electronically via the Mediagnost GmbH website at www.mediagnost.de or will be made available on request via contact@mediagnost.de.

5 SAMPLES

5.1 Sample type

Serum and Plasma
Serum and Heparin/EDTA Plasma yield comparable values.
Serum samples as well as Heparin-, EDTA- and Citrate-Plasma samples are suited. Possible dilution of the sample by the anticoagulant must be considered.

Furthermore, suitable samples are: urine, saliva (low concentration, at least 1:10 dilution), cerebrospinal fluid (at least 1:10 dilution) and cell culture medium (incl. 5% FCS, at least 1:5 dilution). IGF-II concentration in other body fluids or cell culture supernatants can deviate strongly from the serum values.

5.2 Specimen collection

Use standard venipuncture for the blood sampling.
Haemolytic reactions have to be avoided.

5.3 **Required** sample volume: **10 µL**

5.4 Sample stability

Samples should be handled as recommended in general: as fast as possible and chilled as soon as possible. In case there will be a longer period between the sample withdrawal and determination store the undiluted samples frozen -20°C or below in tightly closable plastic tubes. Avoid on principal repeated freeze-thaw cycles of serum/plasma (if required, please subaliquote) although IGF-II levels were found to be unaffected by few cycles in our experiments.

5.5 Interference

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

5.6 Sample dilution

- Dilution: **1:404** with Sample Buffer **SB.**

- **1-step dilution:** Please pipette **2015 µL** Sample Buffer **SB** in PE/PP-Tubes (applicaton of a multi-stepper is recommended in larger series);
subsequently **add 5 µL** sample (dilution 1:404). Incubate at least for 15 Minutes, max. 2 h, use 50 µL in Assay

- **2-step dilution:** Because the pipetting accuracy can rise by the use of 10 µL sample, a 2-step dilution is alternatively possible, for this place **1000 µL** Sample Buffer **SB** in in PE/PP-Tubes, add **10 µL sample** (samples are 1:101 diluted), mix from this solution **50 µL** with **150 µL** Sample Buffer **SB** (samples are 1:404 diluted).

- Incubate at least for 15 Minutes, max. 2 h, use 50 µL in Assay

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-II antibody. Wells are separately breakable.	**(8x12) wells**
CAL A-E	**Calibrators A – E**, lyophilized, (recombinant human IGF-II), concentrations are given on vial labels and on the lot certificate.	**5 x 500 µL**
CTR1	**Control 1,** lyophilised, (human serum), concentration is given on the lot certificate.	**1 x 250 µL**
CTR2	**Control 2,** lyophilised, (human serum), concentration is given on the lot certificate.	**1 x 250 µL**
DET	**Antibody Conjugate,** ready for use, contains coat anti-hIGF-II antibody**.**	**1 x 6 mL**
EC	**Enzyme Conjugate,** ready for use, contains horseradish-peroxidase conjugate to streptavidin.	**1 x 12 mL**
SB	**Sample Buffer,** ready for use, **Please shake before use!**	**1 x 125 mL**
WB	**Washing Buffer,** 20-fold concentrated solution	**1 x 50 mL**
S	**Substrate,** ready for use, horseradish-peroxidase-substrate, stabilised Tetramethylbencidine.	**1 x 12 mL**
STP	**Stop Solution,** ready for use, 0.2 M sulphuric acid.	**1 x 12 mL**
CF	**Cover Foil,** for covering the **microtiter plate.**	**2 x**
	**Instructions for use**	**1 x**
CERT	**Lot Certificate**	**1 x**

6.2 Material is 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 ≥590 nm

7 TECHNICAL NOTES

Storage Conditions

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

Storage Life

The shelf life of the components **after initial opening** is warranted for **4 weeks**, store the unused strips and microtiter wells **airtight** together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The **reconstituted components** calibrators **A-E** and Controls **CTR1** and **CTR2** must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Up to 3 of the freeze-thaw cycles did not influence the assay. The 1:20 diluted Washing Buffer **WB** is 4 weeks stable at 2-8°C

Preparation of reagents

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

Reconstitution

The Calibrators **A – E** and Controls **CTR1 and CTR2** are reconstituted with the 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 Controls **CTR1 and CTR2** with the Sample Buffer **SB** in the same ratio (1:404) as the sample.
The required volume of Washing Buffer **WB** is prepared by 1:20 dilution of the provided 20fold concentrate with Aqua dest.

Assay Procedure

When performing the assay, Blank, Calibrators **A-E**, Controls **CTR1 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** as well as the succeeding Substrate **S** should be added to the plate in the same order and in the same time interval as the samples. Stop Solution **STP** should be added to the plate in the same order as Substrate **S**.
All determinations (Blank, Calibrators **A-E**, Controls **CTR1 and CTR2** and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

Incubation

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

Shaking

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

Washing

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

All washing must be performed with the provided Washing Buffer **WB** diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least.

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

When using an **automatic microtiter** plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Manual washing is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

8 ASSAY PROCEDURE

NOTES: All determinations (Calibrators, Controls and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
When performing the assay, the Calibrators, Controls and the samples should be pipetted as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times **Enzyme Conjugate EC, the Substrate S** as well as the **Stop Solution STP** should be added to the plate in the same order and in the same time interval each, respectively.

1) Add **50 µl Antibody Conjugate DET** in **all** wells used
2) Pipette in positions A1/2 **50 µL Sample Buffer SB**
3) Pipette in positions B1/2 **50 µL of the Calibrator A (0.45 ng/ml),**

   pipette in positions C1/2 **50 µL of the Calibrator B (1.5 ng/ml)**,

   pipette in positions D1/2 **50 µL of the Calibrator C (3 ng/ml)**,

   pipette in positions E1/2 **50 µL of the Calibrator D (5.63 ng/ml)**,

   pipette in positions F1/2 **50 µL of the Calibrator E (9 ng/ml)**.

   To control the correct accomplishment of the assay **50 µL** of the 1:404 (or in respective dilution ratio of the samples) in Sample Buffer diluted **Controls CTR1/CTR2** can be pipetted in positions G1/2 and H1/2.

   Pipette **50 µL** each of the diluted samples (e.g. dilute 1:404 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 **2 hours** at **room temperature** (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** (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** in each well.
10) Incubate the microtiter plate for **30 minutes in the dark** at **room temperature**.
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 ≥ 590 nm)**.

9 EVALUATION OF RESULTS

9.1 Establishing of the calibration curve

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.25, and the absorbance of Calibrator E should be above 1.00. Samples, which yield higher absorbance values than Calibrator E, are beyond the calibration curve, for reliable determinations such samples should be retested at a higher dilution.

The calibrators provided contain the following concentrations of recombinant human IGF-II

Calibrator	CAL A	CAL B	CAL C	CAL D	CAL E
ng/mL	0.45	1.5	3	5.63	9

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-II** concentration in ng/mL of the samples 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	CAL A	CAL B	CAL C	CAL D	CAL E
ng/mL	0	0.45	1.5	3	5.63	9
OD $_{(450\text{-}620\ nm)}$	0.0329	0.127	0.4195	0.8595	1.3695	1.855

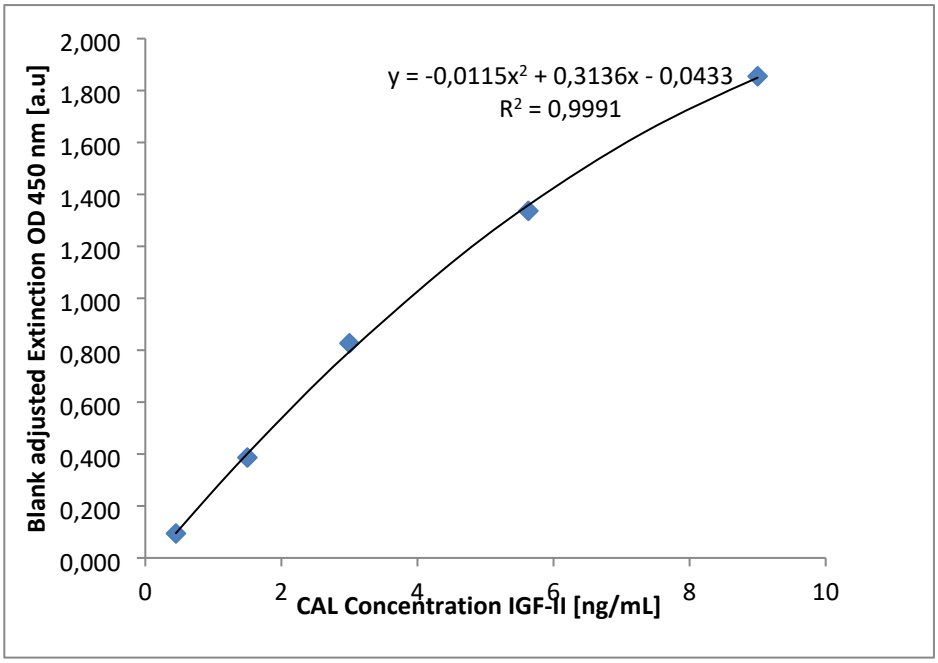

Figure 1 Exemplary calibration curve

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

Exemplary calculation of IGF-II concentrations
Sample dilution: 1:404

Measured extinction of your sample	0.449
Measured extinction of the blank	0.0329

Your measurement program will calculate the IGF-II 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 2nd degree).

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

$y = -0.0115x^2 + 0.3136x - 0.0433$
$0.4161 = -0.0115x^2 + 0.3136x - 0.0433$
$x = 1.49$ ng/mL

If the dilution factor (**1:404**) is taken into account the IGF-II concentration of the undiluted sample is
1.49 ng/mL × 404 = 601.96 ng/mL = 0.602 mg/L

10 EXAMPLARY VALUES

Table 1 Serum levels of IGF-II in ng/ml in healthy persons at various ages*

Percentile			
Age group	**5th**	**50th**	**95th**
Newborns	158	284	516
1-4 weeks	350	486	673
1-6 months	348	551	871
6-12 months	388	582	876
1-3 years	384	596	926
3-5 years	397	617	920
5-7 years	419	638	973
7-9 years	433	656	997
9-11 years	442	662	994
11-13 years	448	671	1006
13-15 years	455	679	1014
15-17 years	452	686	1042
20-30 years	436	679	1058
30-40 years	442	680	1049
40-50 years	407	650	1039
50-60 years	396	644	1049
60-70 years	373	611	1000

*Measurement was performed after acid-ethanol extraction, and values were corrected for recovery (correction factor 1.2). Blum W., 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 (22).

11 PERFORMANCE CHARACTERISTICS

11.1 Sensitivity

Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the 2fold standard deviation of the blank. The mean analytical sensitivity of the E30 is 0.06 ng/mL (Range 0.01 to 0.223, n = 7)

11.2 Specificity

IGF-I, a homologous molecule to IGF-II, was diluted in three different concentrations (250, 750, 1250 ng/mL) and tested with the IGF-II ELISA. None of the samples gave a significant signal.

11.3 Reproducibility and Precision

The Inter- and Intra-Assay variation coefficients are in mean <10%. Exemplary determinations are represented in the Table 2 and 3.

Table 2 Inter-Assay- Variation within one lot measured within 10 months.

	Number [n]	**Mean value [ng/mL]**	**Standard deviation [ng/mL]**	**Variation Coefficient [%]**
Sample 1	18	1019	86.4	8.49
Sample 2	18	692	55.4	8.00
Sample 3	18	570	41.2	7.28

Table 3 Intra-Assay Variation

	Number [n]	Mean value [ng/mL]	Standard deviation [ng/mL]	Variation Coefficient [%]
Sample 1	16	666	20	3.07
Sample 2	16	875	58	6.61
Sample 3	20	621	26	4.17
Sample 4	19	670	41	6.13
Sample 5	20	795	42	5.29

11.4 Linearity

Three serum samples were measured in different dilutions. Standard assay dilution is 1:404. Here sample dilutions of 1:100 – 1:800 were used. Figure 2 demonstrates the linearity of sample dilution. These dilutions cover a concentration range of 4.5 to 0.6 ng/mL.

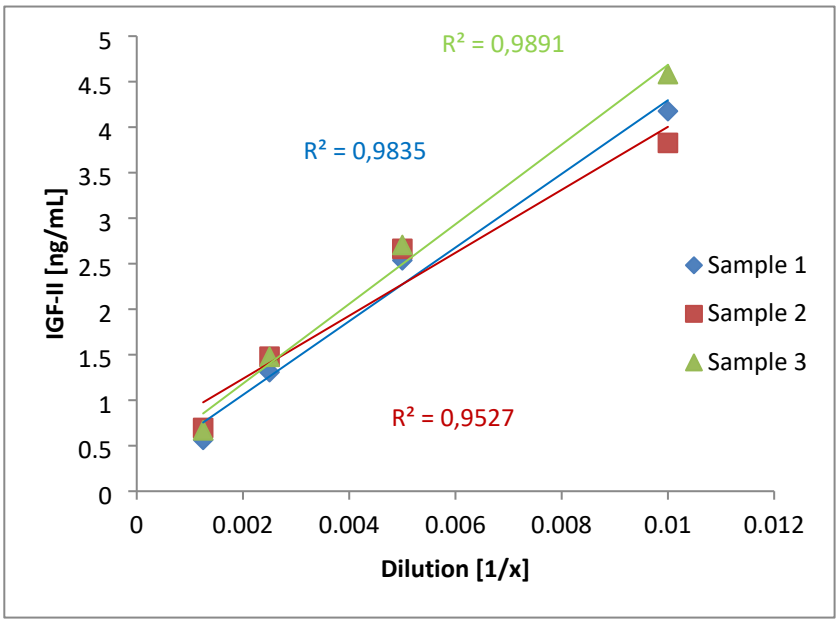

Figure 2 Linearity. Shown are the measured concentrations in different dilutions of three serum samples.

11.5 Interference

Interference of hemoglobin, bilirubin and triglycerides were tested by adding different amounts of these substances to human serum containing IGF-II. For comparison the same amount of buffer without any substance was also added to the serum. Table 4 demonstrates that there is no significant influence of these substances on the measurement of IGF-II in human serum.

Table 4 Interference. Serum samples were enriched with the respective amount of a potentially interfering substance. The recovery in comparison to the non-enriched sample was calculated to assess the influence of haemoglobin, bilirubin and triglycerides on IGF-II measurement.

	Triglyceride 100 mg/mL	Bilirubin 100 µg/mL	Hemoglobin 1 mg/mL
Sample 1	97	103	102
Sample 2	95	95	91
Sample 3	90	111	119

11.6 Matrix tests

Table 5 Results of sample matrix tests 4 ng/mL IGF-II was added to the respectively diluted samples. Enriched samples were measured without further dilution. Shown is the relative recovery of added IGF-II of the value measured in enriched assay buffer.

	IGF-II in the native sample [ng/mL]	IGF-II in the enriched sample [ng/mL]	Recovery [%]
Cerebrospinal fluid			
undiluted	2.86	4.39	31.1
1:2	3.2	5.51	57.8
1:5	3.84	6.78	73.5
1:10	3.03	6.48	**86.3**
Urine			
undiluted	0.36	4.5	**103.5**
1:2	0.35	4.92	**92.9**
1:5	0.35	5.17	**98.0**
1:10	0.35	5.06	**95.7**
Saliva			
undiluted	0.38	3.06	67.0
1:2	0.35	3.99	74.0
1:5	0.24	4.1	78.5
1:10	0.34	4.32	**80.9**
Cell Culture Medium			
undiluted	0.38	3.06	67.0
1:2	0.35	3.99	**91.0**
1:5	0.24	4.1	**96.5**
1:10	0.34	4.32	**99.5**
Cell Culture Medium			
undiluted	0.4	3.62	80.5
1:2	0.4	3.53	78.3
1:5	0.7	5.55	**121.3**
1:10	0.5	5.74	**131.0**

11.7 Cross reactions with animal samples

It has been shown that the test can be used as a heterologous assay for IGF-II measurement in serum samples from bovine and pigs. Species-specific calibration must be carried out by the user.

12 COMPARISON WITH OTHER ASSAYS

The test system was compared with an independent in-house assay, used by the University Tübingen. A good coefficient of determination was measured (R^2=0.8). Passing Bablok regression analysis revealed a regression line described by y =18 +1.3x (RSD 87). The systematic error is negligible and the proportional bias of 30% is probably resulting from usage of different antibodies and calibration material.

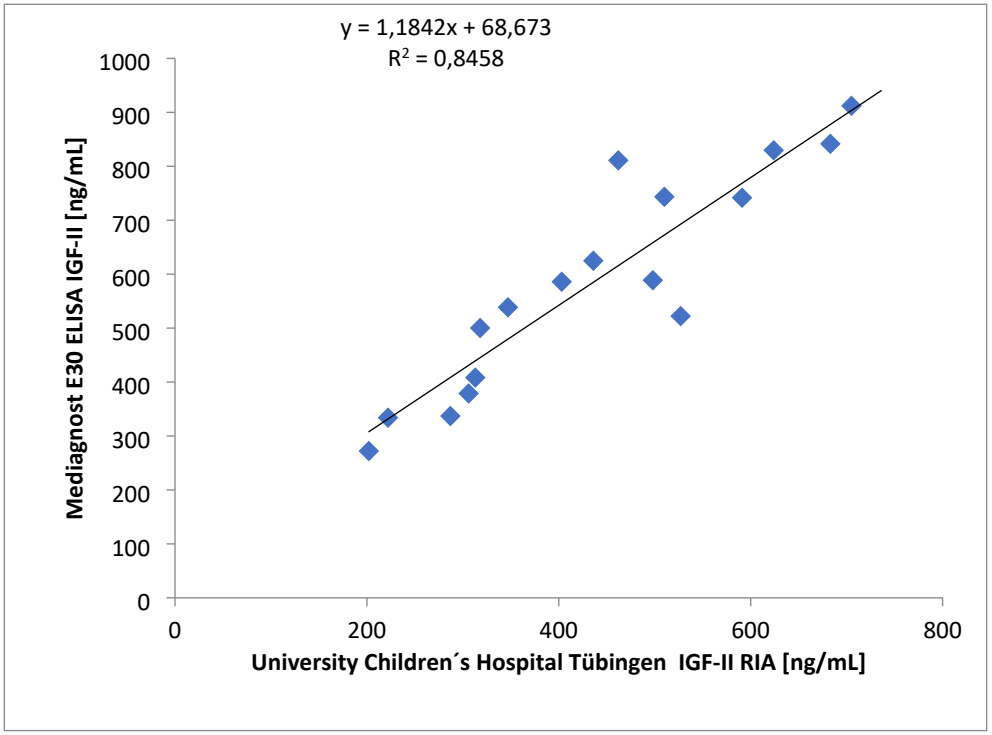

Figure 3 Assay Comparison with an in-house assay of the University Children´s Hospital Tübingen (n = 18).

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14 INTERNATIONAL ASSAY DESCRIPTION

International Test Description

CAL A-E	**Rec in 500 µL SB**	**-**
CTR1	**Rec in 250 µL SB**	**1:404 SB**
CTR2	**Rec in 250 µL SB**	**1:404 SB**
WB 20x	**-**	**1:20 A. dest.** ➔ **WB 1:20**
SPE		**1:404 SB**
↔	**°C 20-25°C** ⏲ **15 min**	
50 µL	**DET**	A1 - End
50 µL	**SB**	A1/A2
50 µL	**CAL A (0.45 ng/mL)**	B1/B2
50 µL	**CAL B (1.5 ng/mL)**	C1/C2
50 µL	**CAL C (3 ng/mL)**	D1/D2
50 µL	**CAL D (5.63 ng/mL)**	E1/E2
50 µL	**CAL E (9 ng/mL)**	F1/F2
50 µL	**CTR1 1:404 SB**	G1/G2
50 µL	**CTR2 1:404 SB**	H1/H2
50 µL	**SPE 1:404 SB**	
TAPE		
⏲ **2 h °C 20-25°C** ↔ **350 rpm**		
5x 300 µL	**5x WB 1:20**	
100 µL	**EC**	
TAPE		
⏲ **0.5 h °C 20-25°C** ↔ **350 rpm**		
5x 300 µL	**5x WB 1:20**	
100 µL	**S**	
⏲ **0.5 h °C 20-25°C**		
STP		
MEASURE		

15 ASSAY PROCEDURE

Preparation of reagents		Reconstitution:	Dilution
CAL A-E	**Calibrators**	in **500 µL** Sample Buffer **SB**	-
CTR1	**Control 1**	in **250 µL** Sample Buffer **SB**	**1:404** with Sample Buffer **SB**
CTR2	**Control 2**	in **250 µL** Sample Buffer **SB**	**1:404** with Sample Buffer **SB**
WB	**Washing Buffer conc.**	-	**1:20** with **Aqua dest.** ➔ **WB 1:20**
Sample and Control dilution: each with Sample Buffer **SB 1:404**, mix immediately, incubate at least for 15 Minutes, max. 2 h. Use **50 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**
50 µL	Antibody Conjugate **DET**	Pipette **in all required number of wells**
50 µL	Sample Buffer **SB** as Blank	A1/A2
50 µL	Calibrator **A** (**0.45 ng/mL**)	B1/B2
50 µL	Calibrator **B** (**1.5 ng/mL**)	C1/C2
50 µL	Calibrator **C** (**3 ng/mL**)	D1/D2
50 µL	Calibrator **D** (**5.63 ng/mL**)	E1/E2
50 µL	Calibrator **E** (**9 ng/mL**)	F1/F2
50 µL	Control **CTR1** (1:404 diluted)	G1/G2
50 µL	Control **CTR2** (1:404 diluted)	H1/G2
50 µL	Sample **SPE** (1:404 diluted)	in the rest of the wells according the requirements
Cover the wells with the cover foil.		
Sample Incubation: 2 h at 20-25°C, 350 rpm		
5 x 300 µL	Aspirate the contents of the wells and **wash** 5 x with **300 µL** each Washing Buffer **WB 1:20** **/well**	In each well
100 µL	Enzyme Conjugate **EC**	In each well
Cover the wells with the cover foil.		
Incubation: 30 Minutes at 20-25°C, 350 rpm		
5 x 300 µL	Aspirate the contents of the wells and **wash** 5 x with **300 µL** each Washing Buffer **WB 1:20** **/well**	In each well
100 µL	Substrate **S**	In each well
Substrate S Incubation: 30 Minutes in the Dark at 20-25°C		
100 µL	Stop Solution **STP**	In each well
	Measure the absorbance within **30 Minutes** at **450 nm** with ≥ 590 nm as reference wavelength.	