BioVendor
Research
and Diagnostic Products

Human IGF-II ELISA

(Insulin Like Growth Factor-II)

Cat. No.: RMEE30

IVD CE

TECHNICAL FEATURES+APPLICATIONS

- Measurement of IGF-II in **non-extracted** serum and plasma samples
- Calibrated against the **International Standard: WHO NIBSC 96/538**
- No interference by IGF-binding proteins through excess IGF-I
- Precise measurement of very low IGF-II levels: high **sensitivity of 0.02 ng/ml**
- Inter- and Intra-Assay variance: maximal 7.2 and 6.6%

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. Its 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.

INTENDED USE

Scientific investigations in the field of neonatal hypertrophy (IGF-II is a foetal growth factor) and malignancies (IGF-II is an monogenic growth factor). Age dependent reference values are shown in Table 4.

IGF-II seems to be of use in differential diagnostics of malignancies. Thus, it is possible to differentiate by IGF-II between adrenocortical carcinomas and adenomas (24). Further tumor staging and differentiation between hyperplasia and carcinoma can be improved by IGF-II measurements in prostate tumors (25). The IGF-System seem to be of relevance in neurodegeneration as well, e.g. Alzheimer´s and Parkinson's diseases (26).

PRINCIPLE

The Mediagnost ELISA for IGF-II 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

PERFORMANCE CHARACTERISTICS AND VALIDATION

The IGF-II ELISA is calibrated against the **International Standard: WHO NIBSC 96/538**

The standards of the ELISA are **human IGF-II** in concentrations **0.45; 1.5; 3; 5.63 and 9 ng/ml**, respectively the assay range covers –at recommended normal sample dilution- the **range to 2400 ng/ml**. By varying the sample dilution this can be adapted to the special individual requirements.

Sensitivity

The **analytical sensitivity** of the ELISA yields **0.02 ng/ml** (2 SD of zero standard in 20fold determination).

The Inter- and **Intra-Assay variation** coefficients are less than 7.2% and 6.6 % respectively. Exemplary determinations are represented in the Table 1 and 2.

Table 1: Inter-Assay-Variation (n=12)

	Mean value (ng/ml)	Standard deviation ng/ml)	Variation Coefficient (%)
Sample 1	381.53	27.54	7.22
Sample 2	817.81	57.70	7.06
Sample 3	639.41	45.47	7.11

Table 2: Intra-Assay-Variation (n=16)

	Mean value (ng/ml) ng/ml)	Standard deviation (ng/ml)	Variation Coefficient %)
Sample 1	666.38	20.45	3.07
Sample 2	875.22	57.89	6.61

Because of the high specificity of the used antibodies the endogenous and structurally very similar IGF-I existing in variable concentration in the samples does not affect the correctness of the IGF-II determination up to 1000 ng/ml (s.Table 3).

Table 3: Dependence of the determined IGF-II concentration on the IGF-I content of the samples

Addition GF-I (ng/ml)	Serum Sample ng/ml)	NIBSC 96/538 in Samplebuffer (ng/ml)
0	603	3,49
100	630	3,39
250	605	3,44
500	614	3,59
1000	604	3,68

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

Serum samples as well as Heparin-, EDTA- and Citrat-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 (inc. 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.

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.

The IGF-II ELISA makes the correct determination possible of the samples starting from a dilution of 1:401 over a far concentration range.

The high sensitivity of the assays allows IGF-II determinations in small sample volumes, which is limited by pipetting accuracy rather than the amount of IGF-II. Generally a serum or plasma dilution of **1:401 is very well suited**. However, the respective suitable dilution should be examined first.

Suggestion for dilution protocol:

Please pipette 2000 µl **Sample Buffer PP** in PE/PP-Tubes (applicaton of a multi-stepper is recommended in larger series); subsequently add 5 µl serum or plasma samples (dilution 1:401). After mixing **use 50 µl of this dilution per determination**.

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 PP 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 (samples are 1:404 diluted). After mixing **use 50 µl of this dilution per determination.**

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 anti-human IGF-II Antibody, packed in a laminate bag.
2)		**Standards A-E**, lyophilised, contain human IGF-II. Standard values are **between 0.45 and 9 ng/ml (0,45; 1,5; 3; 5,63; 9 ng/ml)** IGF-II and have to be reconstituted in **500 µl (each) in Sample Buffer PP**. 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. 50 µl per well are used in the assay
4)	BUF PP	**Sample Buffer PP**, 125 ml, ready for use, please use for reconstitution of Standards and Controls and for dilution of samples and Controls
5)	Control	**Control Serum KS1, KS2**, 250 µl, lyophilised, contain human serum and has to be reconstituted in 250 µl **Sample Buffer PP**. The reconstituted Control Sera must be stored in the original flask as soon as possible at –20°C after using. When using 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. The IGF-II target value concentrations and the respective ranges are given on the vial labels. The dilution of the Control Sera should be according to the dilution of the respective samples.
6)	Ab	**Antibody Conjugate AK**, 6 ml, ready for use, contains the biotinylated anti-IGF-II antibody. Use 50 µl for each well in the assay.
7)	CONJ	**Enzyme Conjugate EK**, 12 ml, ready for use, contains horseradisch-peroxidase conjugate to streptavidin, use 100 µl for each well in the assay.
8)	WASHBUF 20x	**Washing Buffer (WP), 50 ml, 20X 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.
9)	SUBST	**Substrate (S)**, 12 ml, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H2O2 Tetramethylbencidine.
10)	H2SO4	**Stopping Solution (SL)**, 12 ml, ready for use, 0.2 M sulphuric acid, Caution acid!
11)		Sealing tape for covering of the microtiter plate, 2 x, adhesive.

MATERIALS REQUIRED BUT NOT PROVIDED

Precision pipettes (50 and 100 µl), Micropipettes and multichannel pipettes with disposable plastic tips

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

Vortex-mixer

Device to aspirate the standards and the samples from the wells (recommended because of the potential danger of infection by human samples)

Timer (120 min. range)

Reservoirs (disposable)

Plate washer and plate shaker (recommended)

Calibrated Micro plate reader ("ELISA-Reader") with filter for 450 and 620nm (or ≥590 nm)

Technical Recommendations

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.

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

Standards and Controls

For the reconstitution of the lyophilised components (Standards A - E and Control Sera KS1 & KS2) the kit Sample Buffer PP 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 should result) with a Vortex mixer.

The reconstituted standards and controls can be stored for 3 months at –20°C. Repeated freeze/thaw cycles have to be avoided. 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.

Washing Buffer

The required volume of washing buffer is prepared by 1:20 dilution of the provided 20-fold concentrate with deionised water. The diluted Washing Buffer is stable for max. 4 weeks at 2-8°C.

Substrate Solution

The **Substrate Solution S**, stabilised H2O2-Tetramethylbencidine, is photosensitive – store and incubate in the dark.

Microtiter plate

Store the once unused microtiter strips and wells together with the desiccant in the tightly closed clip lock bag at 2 – 8°C use in the frame provided. The labelled expiry is not influenced in case of proper storage.

WARNINGS AND PRECAUTIONS

For in-vitro diagnostic use only. For 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. The Mediagnost GmbH is not liable for any loss or harm caused by non-observance of the instructions, as far as no law withstands.

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

Caution: This kit contains material of human and/or animal origin.

Human Serum

Contained in following components: **Control Serum KS1 and KS2**.

The sources of human sera were 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 patient's specimens should be treated as potentially infectious.

Stop solution contains 0.2 M Sulfuric Acid (H_2SO_4)

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

2-Methyl-4-Isothiazolin-3-one

contained in following components: AK, EK, VP, PP

< 0.01%	2-Methyl-4-isothiazolin-3-one Solution
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

5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-Isothiazol-3-one

contained in following components: **AK, EK, VP, WP, PP**

< 0.01% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-Isothiazol-3-one

Solution

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	After contact with skin, wash immediately with plenty of water

TMB-Substrate (S) contains 3,3´,5,5´ Tetramethylbenzidine.

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.

ASSAY PROCEDURE

NOTES: All determinations (Standards, Control Sera 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). To avoid distortions due to differences in incubation times **Enzyme Conjugate EK, the Substrate Solution S** as well as the **Stop Solution SL** should be added to the plate in the same order and in the same time interval each, respectively.

It is recommended to use the microtiter plate shaker, if this is not available, please use the alternative procedure (see below).

1) Add **50 µl Antibody Conjugate AK** in **all** wells used

2) Pipette in positions A1/2 **50 µl Sample Buffer PP**

3) Pipette in positions B1/2 **50 µl of the Standard A (0.45 ng/ml)**,
pipette in positions C1/2 **50 µl of the Standard B (1.5 ng/ml)**,
pipette in positions D1/2 **50 µl of the Standard C (3 ng/ml)**,
pipette in positions E1/2 **50 µl of the Standard D (5.63 ng/ml)**,
pipette in positions F1/2 **50 µl of the Standard E (9 ng/ml)**.
To control the correct accomplishment of the assay **50 µl** of the 1:401 (or in respective dilution ratio of the samples) in Sample Buffer diluted **Control Sera KS1/KS2** can be pipetted in positions G1/2 and H1/2 .
Pipette **50 µl** each of the diluted samples (e.g. dilute 1:401 with Sample Buffer **PP**) 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 **250 µl Washing Buffer WP / well**.

6) Following the last washing step pipette **100 µl** of the **Enzyme Conjugate EK** in each well.

7) Cover the wells with sealing tape and incubate the plate for **30 Minutes** at **room temperature** (if possible shake ≥350 rpm).

8) After incubation wash the wells 5 times with Washing Buffer **WP** as described in step 5.

9) Pipette **100 µl** of the **Substrate Solution 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 Stopping Solution SL** to all wells.

12) Measure the absorbance within **30 minutes at 450 nm (Reference filter ≥ 590 nm; e.g. 620 nm)**.

CALCULATION OF RESULTS

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.20, and the absorbance of standard E should be greater than 1.00.

Samples, which yield higher absorbance values than Standard E, are beyond the standard curve, for reliable determinations such samples should be retested at a higher dilution.

Establishing the Standard Curve

The standards provided contain the following concentration of recombinant hIGF-II:

Standard	**A**	**B**	**C**	**D**	**E**
ng/ml	0.45	1.5	3	5.63	9

1) Calculate the **mean absorbance** (MA) value for the blank from the duplicated determination (well A1/A2).

2) Subtract the mean absorbance (MA) 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 on semi-log paper (lin-log).

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

5) The **IGF-II concentration in ng/ml** of the samples can be calculated **by multiplication** with the **respective dilution factor**.

EXPECTED VALUES

Table 4: 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).

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SUMMARY –IGF-II ELISA RMEE30

Reconstitution / Dilution of Reagents		
Standards A-E	Reconstitution in **Sample Buffer PP**	**500 µl**
Control Serum KS1	Reconstitution in **Sample Buffer PP**	**250 µl**
Control Serum KS2	Reconstitution in **Sample Buffer PP**	**250 µl**
Wash Buffer WP	dilute in **A. dest**. (eg. total volume of 50 ml in a graduated flask and fill up to 1000 ml)	**1:20**
Sample + Control Sera KS1 and KS2: dilute 1:401 in Sample Buffer PP, mix immediately, incubate at least for 15 min, max. 2h. Use 50 µl for each well in the assay.		
Before conducting the assay equilibrate all reagents to room temperature.		

Assay Procedure for Double Determinations:

Pipette	Reagent	Position
50 µl	Antibody Conjugate **AK**	in **all** wells used
50 µl	Sample Buffer **PP (blank)**	A1 and A2
50 µl	Standard **A (0.45 ng/ml)**	B1 and B2
50 µl	Standard **B (1.5 ng/ml)**	C1 and C2
50 µl	Standard **C (3 ng/ml)**	D1 and D2
50 µl	Standard **D (5.63 ng/ml)**	E1 and E2
50 µl	Standard **E (9 ng/ml)**	F1 and F2
50 µl	Control Serum **KS1**	G1 and G2
50 µl	Control Serum **KS2**	H1 and H2
50 µl	Samples	following wells
Cover the wells with the sealing tape.		

Incubation: 2 h at RT, ≥ 350 rpm

5x 250 µl	Aspirate the contents of the wells and wash **5x** with **250 µl Wash Buffer WP**	each well
100 µl	Enzyme Conjugate **EK**	each well

Incubation: 30 min at RT, ≥350 rpm

5x 250 µl	Aspirate the contents of the wells and wash **5x** with **250 µl** Wash Buffer WP	each well
100 µl	Substrate **S**	each well

Incubation: 30 min in the dark **RT**

100 µl	Stop Solution **SL**	each well
Measure the absorbance within **30 min** at **450 nm** with **≥ 590 nm** as reference wavelength.		

REF RMEE30 **International Test Description**

CAL A-E	**A -E**	**Rec in 500 µl BUF PP**	
Control	**KS1&KS2**	**Rec in 250 µl BUF PP**	
WASHBUF 20x	**WP**		**1:20 DILU A. dest.**

SPE + Control 1:401 DILU BUF PP	↔	**0.25 h**
°C 20-25 °C		

50 µl	**Ab**	A1 - End
50 µl	**BUF PP**	A1/2
50 µl	**CAL A Std A (0.45 ng/ml)**	B1/2
50 µl	**CAL B Std B (1.5 ng/ml)**	C1/2
50 µl	**CAL C Std C (3 ng/ml)**	D1/2
50 µl	**CAL D Std D (5.63 ng/ml)**	E1/2
50 µl	**CAL E Std E (9 ng/ml)**	F1/2
50 µl	**CONTROL KS 1**	G1/2
50 µl	**CONTROL KS 2**	H1/2
50 µl	**SPE**	
	TAPE	

2 h °C 20-25 ≥ 350 rpm ↔

5x 250 µl	**5x WASHBUF WP**
100 µl	**CONJ**
	TAPE

0.5 h °C 20-25 ≥ 350 rpm ↔

5 x 250 µl	**5x WASHBUF WP**
100 µl	**SUBST TMB S**

30 min °C 20-25

100 µl	**H2SO4 SL**
	MEASURE

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