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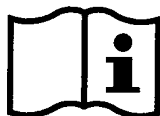

User´s Manual

IGFBP-2 ELISA

Enzyme Immunoassay for Quantitative Determination of

Insulin-like Growth Factor Binding Protein-2

IVD

REF DEE005

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TABLE OF CONTENTS

TECHNICAL FEATURES 3
CLINICAL IMPLICATIONS 3
INTRODUCTION 3
PHYSIOLOGICAL MEANING 4
INTENDED USE 4
PRECAUTIONS 4
METHODOLOGY 5
- Assay Characteristics and Validation 5
- Clinical Validation 6
- Sample Preparation and Storage 7

MATERIALS 7
- Materials Provided 7
- TECHNICAL HINTS 8
- Materials not Provided 8

ASSAY PROCEDURE 9
EVALUATION OF RESULTS 10
- Establishing the Standard Curve: 10

EXPECTED VALUES 10
LIMITATION 10
REFERENCES 13
SUMMARY OF THE ASSAY PROCEDURE: 15

TECHNICAL FEATURES

- Highly specific and sensitive assay for quantitative detection of IGFBP-2 in human serum and in other human body fluids
- Recombinant standard
- Contains antibodies against complete human IGFBP-2 (1,2)
- Detection limit 0.2 ng/ml
- No sample extraction is required
- Complete recovery
- High stability of IGFBP-2 upon storage, resistent against at least 10 thawing and freezing cycles.

CLINICAL IMPLICATIONS

The IGFBP-2 concentration is age-dependent in blood (3).

Normal values for healthy individuals (1.5 to > 70 years) were evaluated for this assay.

Supplementary parameter to IGFBP-3 in the diagnosis of growth disorders (IGFBP-2/IGFBP-3 ratio), IGFBP-2 is an inhibitor of growth hormone action (3,4).

Progression-dependent tumor marker in leukaemia (5), astrocystic CNS tumors (6,7), prostate- (8), suprarenal cortex-(9)-, hepatocellular (10) and other carcinomas.

Anti-aging parameter: IGFBP-2 as a marker of physiological functionality (20).

INTRODUCTION

Insulin-like growth factors (IGFs) regulate the proliferation, differentation, apoptose, cell adhesion and metabolism in various tissues and cell types. The IGF-I, which is produced mainly in liver under the influence of growth hormone (GH), regulates as hormone the linear growth of the bones and the process of sexual maturity, while IGF-II is mainly a growth factor of fetal tissue (11-13). The biological actions of IGF over the IGF-Type-I receptor are modulated variably through the IGF binding proteins (IGFBP-1 to-6) (14). IGFBP-2 is, after IGFBP-3, the second most frequent IGFBP in the human blood. IGFs, especially tumor typical pro-IGF-forms and hormones regulate the expression of IGFBP-2, GH effect is thereby inhibiting. At cellular level IGFBP-2 seems to stimulate the proliferation and dissemination of solid tumors via an IGF-independent mechanism (15,16).

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PHYSIOLOGICAL MEANING

IGFBP-2 is a unglycosylated polypetide of 31.3 kDa, which forms binary IGF-complexes and shows no circadian rhythm in the circulation.The serum concentration of IGFBP-2 increases in fasting, after major surgery and after trauma, but the increasing of the concentration is most intensive in malignant diseases. The correlation of the IGFBP-2 level to the degree of progression is a striking feature in various tumor types as is the normalization of the IGFBP-serum levels after remission (5-10). During the GH-therapy, e.g. in short stature and in GH-abuse (doping) the IGFBP-2 level decreases. In Trisomy 18 IGFBP-2 in maternal serum is decreased and IGFBP-1 is increased; therefore the ratio IGFBP-2 /IGFBP-1 is a marker for this chromosome abnormality (17).

Low IGFBP-2 serum levels were found to be in a study as a significant indicator for good physical-functional condition of senior men (positive: muscle power bzw. Bone density; negative: adipose mass), (20).

INTENDED USE

This IGFBP-2 Enzyme Immunoassay-Kit is suited for quantitative determination of IGFBP-2 in human serum for diagnostic and scientific purposes. Highly Elevated IGFBP-2 levels, which are far over the reference ranges (s. table 5+6) can indicate, in correlation to the metastatic spread, a metastasising tumor. IGFBP-2 can be measured as an additional parameter while monitoring a tumortherapy (e.g. chemotherapy) and as an indicator for trisomy 18 (17) in maternal blood.

PRECAUTIONS

The kit should not be used beyond the expiration date on the kit label.

All reagents are for in vitro use only!

In conducting the assay, follow strictly the test protocol. The acquisition, possession and use of the kit is subject to the regulations of the national regulatory authorities.

Reagents with different lot numbers should not be mixed.

Reagents contain as preservatives
ProClin 950
Following components contain ProCline 950 : **A-E, AK, VP**
< 0,1% 2-Methyl-4-isothiazolin-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

Kathon CG
Following components contain Kathon CG: **A-E, AK, VP, WP**
< 0,1% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one und 2-methyl-2H-isothiazol-3-one

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

Stop solution contains 0.2 M Sulfuric Acid (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.

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

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

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 throughly with water. Immediately see a physician.The Stop Solution provided is an acid solution. Avoid direct contact. Wear eye, hand, face and clothing protection when using this material.

The handling of potentially infectious material must comply with the following guidelines:

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.

METHODOLOGY

Assay Characteristics and Validation

The ELISA for IGFBP-2 utilizes a specific high affinity polyclonal antibody and a specific monoclonal antibody for this protein. It recognizes quantitatively IGFBP-2 and is unaffected by excess of IGF-I or IGF-II levels. Related molecules such as IGFBP-3 show no cross-reaction in the assay. The standards are also prepared of recombinant IGFBP-2 in the range of 1 to 80 ng/ml. Detailed specification of antibody and pure IGFBP-2 (data sheets) can be ordered from Demeditec Diagnostics GmbH.

Chromatographically purified IGFBP-2, as well as IGFBP-2-antiserum are available at Demeditec Diagnostics GmbH also as research reagents.

Different human sera were spiked with recombinant human IGFBP-2:recovery was found between 97% and 115%.

The theoretical sensitivity of the assay is 0.2 ng/ml (2 x SD of zero standard). Intra-assay and inter-assay variation coefficients were found both < 10%. Exemplary determinations are shown in the tables 1 and 2.

Table 1 : Interassay-Variation

Sample1 (ng/ml)	137	159	152
Sample 2 (ng/ml)	672	697	688
Sample 3 (ng/ml)	928	929	956

Table 2: Intra-Assay-Variation

Sample 1 ng/ml	322	375	298	305	318	311	320	325	302	301	305	317
Sample 2 ng/ml	612	609	616	648	594	597	620	613	617	611	636	698

Table 3: Linearity of the sample dilution:

Dilution	**Serum 1 (ng/ml)**	**Serum 2 (ng/ml)**	**Liquor (ng/ml)**	**Amniotic fluid (ng/ml)**
1:10	938	582	426	Not determined
1:20	1061	673	428	460
1:40	1055	719	379	483
1:80	1004	691	318	431
1:160	952	668	426	415

Clinical Validation

Clinical validation was achieved by determination of IGFBP-2 levels in 400 normal children and adults (see table 6 and figure 1) and children with growth disorders (GH deficiency, Ullrich-Turner syndrome, idiopathic short stature).

Sample Preparation and Storage

The stability of IGFBP-2 makes sample preparation unproblematic. Blood samples may be taken at any time of the day. Whole blood should be processed within two hours. Once separated the samples should be stored frozen until measurement. IGFBP-2 levels are only modestly influenced by improper handling or storage and remain stable over several days at elevated temperatures in normal and in many clinical situations. Store undiluted samples frozen in a plastic vial. Repeated freezing and thawing of serum/plasma seems to have an measurable effect on IGFBP-2 levels only after >10 cycles.

The high sensitivity of the assay allows measurement of IGFBP-2 in small sample volumes which is limited by pipetting accuracy rather than the amount of IGFBP-2.

Serum samples should be diluted prior to measurement 1:10-30-fold with **Dilution Buffer VP** depending on the expected values. In general a dilution of 1:21 is appropriate (and herewith is the minimal essential sample volumen for a double determination: 15 µl serum). Sample extraction is not required.

Suggestion for dilution protocol:

Mix 15 µl serum manually or with the aid of a dilutor with 300 µl **Dilution Buffer VP** (1:21). Use 2 x 100 µl of this dilution in the assay or pipette 100 µl buffer in wells and add 5 µl serum.

IGFBP-2 concentrations may be completely different in body fluids of human origin other than serum or cell culture supernatant (s.Table 4).

MATERIALS

Materials Provided

1) **Microtiter plate**, ready for use: **Microtiter plate** with 96 wells, divided up in 12 strips with 8 wells separately breakable, coated with anti-IGFBP-2 antibody and packed in a laminate bag.

2) **Standards A-E,** lyophilized: contain recombinant IGFBP-2: Standard values are between 1 - 80 ng/ml (1, 10, 20, 40, 80 ng/ml) IGFBP-2 and have to be reconstituted with **750 µl Dilution Buffer VP** each.

3) **Controlserum KS,** lyophilized: Contains human serum and has to be reconstituted with **100 µl** Dilution Buffer **VP**. The exact concentration of IGFBP-2 is given on the vial label.

4) **Dilution Buffer VP**, 50 ml, ready for use.

5) **Antibody-POD-Conjugate AK,** 12 ml, ready for use, contains a mixture of biotinylated anti-IGFBP-2 antibody and HRP (Horseradish Peroxidase)-labelled Streptavidin. Use 100 µl/well in the assay.

6) **Washing Buffer WP**, 50 ml, 20-fold concentrated: The **Washing Buffer** has to be diluted 1:20 with distilled water before use.

7) **TMB-Substrate Solution** S 12 ml, ready for use.

8) **Stopping Solution SL**, 0.4 N sulphuric acid, 12 ml, ready for use. *Caution!*

9) **Sealing tape** for covering of the microtiter plate, 2 x

TECHNICAL HINTS

The Microtiterplate and all reagents are stable until the expiry date if stored in the dark at 2-8°C (s.label). Store the unused seal stripes of the microtiterplate together with the desiccant at 2-8°C.

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

The 1:20 diluted Washing Buffer WP is stable only limited. Please dilute only according to requirements.

For reconstitution of lyophilized components the kit **Dilution Buffer VP** should be used. It is recommended to keep reconstituted reagents at room temperature for half an hour, and then to mix them throughly but gently (no foam should result), e.g., with a Vortex mixer.

After reconstitution components should be stored at 2-8°C for up to 1 week. If longer storage time is needed, store the components frozen at -20°C or below. Reconstituted standards (**A-E**) and Control Serum (**KS**), are stable at -20°C at least 2 months. Avoid repeated freeze-thaw cycles. In case you plan to to perform multiple independent determinations over a longer period with one kit, you should aliquot the components prior to freezing into suitable smaller volumes. This is strongly recommended.

Room temperature incubation means: incubation at 20 - 25°C.

When performing the assay, the **Standards (A-E)**, **Control Serum (KS)** should be pipetted as fast as possible (e.g., 15 Minutes). Antibody-POD-Konjugat **(AK)** as well as the succeeding **Substrate solution S** should be added to the plate in the same order and the same time interval as the samples. **Stop Solution (SL)** should be added to the plate in the same order as the **Substrate Solution (S).**

Materials not Provided

- Distilled or demineralized water for dilution of the washing buffer W
- Micropipettes and multichannel pipettes with disposable plastic tips
- Vortex-mixer
- Device to aspirate the standards and the samples from the wells
- Plate washer and plate shaker (recommended)
- Microplate reader ("ELISA-Reader") with filter for 450/620nm wavelength
- Foil welding device for laminate bags (recommended)

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ASSAY PROCEDURE

Samples (standards and patient specimen) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

1) add **100µl dilution buffer VP** in wells A1/A2 (blank) and
2) pipette in positions B1/B2 **100µl standard A,**
   pipette in positions C1/C2 **100µl standard B,**
   pipette in positions D1/D2 **100µl standard C,**
   pipette in positions E1/E2 1**00µl standard D,**
   pipette in positions F1/F2 **100µl standard E.**

To control correct accomplishment **100 µl** of the **1:21** diluted **control serum KS** can be pipetted in positions G1/2.

Pipette **100 µl** of the **diluted sample** in the rest of the wells, according to requirements.

3) Cover the wells with sealing tape and incubate the plate for **1 hour** at **room temperature** (if possible, shake at 350 rpm).
4) After incubation aspirate the contents of the wells into a disinfectant (risk of infection!) and wash the wells 3 times with **250 µl** of **Washing Buffer WP** / well respectively. The **Washing Buffer** should incubate at least for **15 seconds**/well.
5) Following the last washing step pipette **100µl** of the **Antibody-POD-Conjugate AK** in each well.
6) Cover the wells with sealing tape and incubate the plate for **30 Minutes** at **room temperature** (if possible shake ≥350 rpm).
7) After incubation wash the wells 3 times with **Washing Buffer WP** as described above.
7) Pipette **100 µl** of the **TMB-substrate** solution **S** in each well.
8) Incubate the plate for **15 minutes** in the dark at **room temperature**.
9) Stop the reaction by adding **100 µl** of **Stopping Solution SL** to all wells.
10) Measure the absorbance within **30 minutes** at **450 nm** (reference filter: **≥ 590 nm**).

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EVALUATION OF RESULTS

Establishing the Standard Curve:

The standards provided contain the following concentrations of IGFBP-2 :

Standard	A	B	C	D	E
ng/ml	1	10	20	40	80

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 absorbance of all other values.
3) Plot the standard concentrations A-E on the x-axis versus the mean value of the absorbance of the standards on the y-axis. By using the mean absorbances of the samples herewith the sample concentrations can be recieved.
4) Recommendation: Calculation of standard curve and sample concentrations should be done by using a computer programme, because the standard curve is in general best described by non-linear regression or a higher-grade polynom.
5) The IGFBP-2 concentration in ng/ml of the samples can be calculated by multiplication with the respective dilution factor.

EXPECTED VALUES

The normal ranges of IGFBP-2 serum levels are log-normally distributed, depended on age (Table 6) and depended on Body Mass Index (BMI; Table 5) .

Table 4 : Expected values of IGFBP-2 in body fluids of human origin and in cell culture supernatants:

Sample	Expected Value (ng/ml)
serum	[100 - 1000]
liquor	[100 - 300]
amniotic fluid	[200 - 10000]
seminal plasma	[5000 - 15000]
breast milk	[1500-3000]
cell culture supernatants	[5 - 300]

LIMITATION

Deviation from the reference range can be expected especially in hypothyroidism, after major surgery, in polytrauma, in Diabetes mellitus (due to insulin therapy), in fasting and in malignant diseases.

Table 5.: BMI dependent reverence values, adults between 20 and 80 years

BMI $[kg/m^2]$	N	IGFBP-2 [ng/ml] mean	SD	percentiles 5th	50th	95th
15	12	612	110	431	612	793
17,5	14	568	126	361	568	775
20	76	509	144	271	509	746
22,5	124	449	162	182	449	716
25	101	398	165	127	398	670
27,5	52	348	147	106	348	590
30	25	315	118	120	315	510
32,5	15	282	90	135	282	430
35	4	251	80	119	251	383
37,5	4	220	71	104	220	336

Table 6: IGFBP-2 serum levels (in ng/ml) of > 400 healthy individuals. The normal range is given by the 5., 50. and 95. percentile for age classes.

Age-dependent normal range of serum IGFBP-2

age (years)	5. percentile (ng/ml)	50. percentile (ng/ml)	95. percentile (ng/ml)
1	408	545	728
2	359	500	696
3	317	460	668
4	277	421	640
5	243	388	617
6	217	361	602
7	194	336	583
8	173	312	562
9	154	289	542
10	138	268	522
11	123	249	503
12	111	232	486
13	101	219	477
14	94	212	470
15	89	207	465
16	86	207	460
17	84	214	466
18	84	223	483
19	84	232	500
25	99	280	580
35	110	381	686
45	130	403	702
55	140	410	715
65	151	418	727
75	153	427	740
80	156	430	744

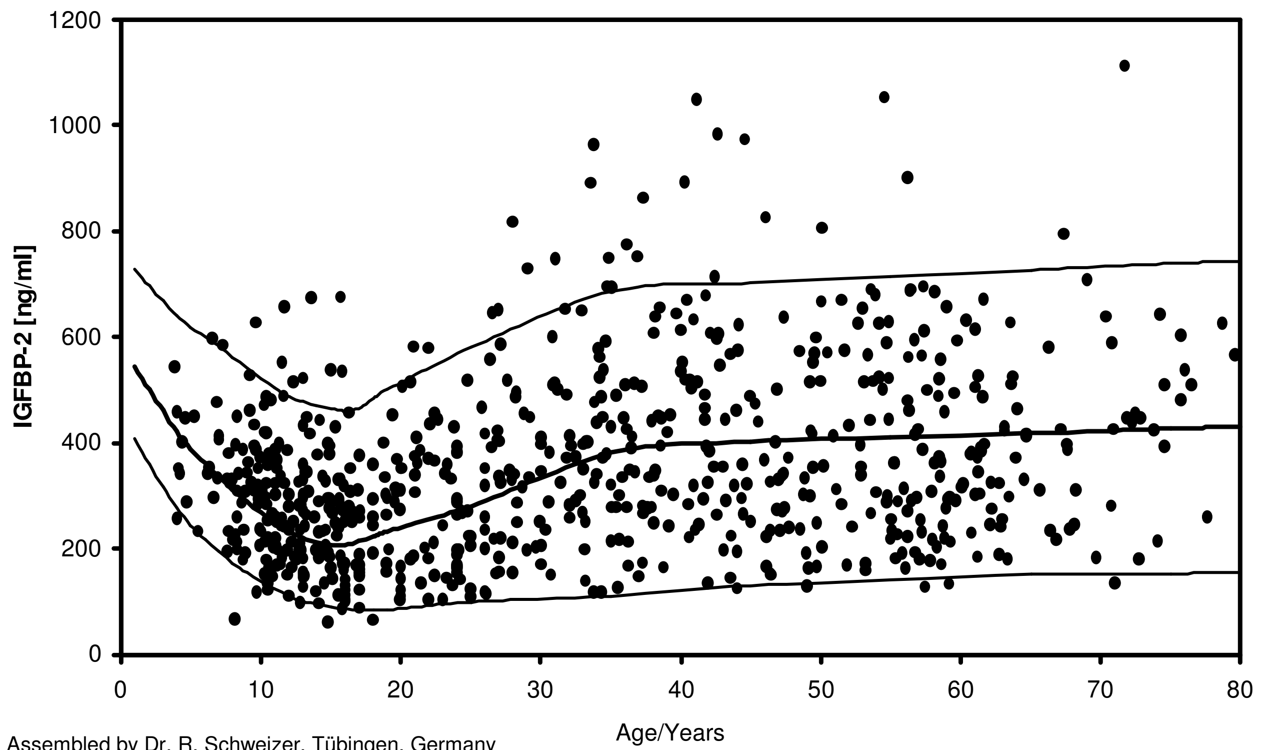

Fig. 1: IGFBP-2 serum levels (in ng/ml) of > 400 healthy individuals. The normal range is given by the 5th, 50th and 95th percentile.

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SUMMARY OF THE ASSAY

Reagent:	Reconstitution:	dilution:
Standards A-E	in **750 µl** Dilution Buffer **VP**	
Control Serum KS	in **100 µl** Dilution Buffer **VP**	**1:21 with** Dilution Buffer **VP**
Washing Buffer WP		**1:20** with **Aqua. dest.** (e.g., add the complete contents of the flask **(50 ml)** into a graduated flask and fill with **A.dest.** to 1000 ml).
Sample Dilution: Serum samples should be diluted prior to measurement 1:10-30-fold with **Dilution Buffer VP** depending on the expected values. In general a dilution of 1:21 is appropriate. Use 2 x 100 µl of this dilution in the assay		

Assay Procedure for Double Determination

Pipette	Reagents	Position
100 µl	Dilution Buffer **VP**	A1/2
100 µl	Standard **A** (**1 ng/ml**)	B1/2
100 µl	Standard **B** (**10 ng/ml**)	C1/2
100 µl	Standard **C** (**20 ng/ml**)	D1/2
100 µl	Standard **D** (**40 ng/ml**)	E1/2
100 µl	Standard **E** (**80 ng/ml**)	F1/2
100 µl	Controll Serum **KS**	G1/2
100 µl	Sample dilution	following wells
Cover the wells with the sealing tape.		
Incubation: 1 h at RT, ≥ 350 upm		
3x 250 µl	Aspirate the contents of the wells and wash **3x** with **250 µl Wash Buffer WP**	each well
100 µl	**Antibody-POD-Conjugate AK**	each well
Incubation: 30 min at RT, ≥ 350 rpm		
3x 250 µl	Aspirate the contents of the wells and wash **3x** with **250 µl Wash Buffer WP**	each well
100 µl	Substrate Solution **S**	each well
Incubation: 15 min in the Dark at RT		
100 µl	Stopping Solution **SL**	each well
Measure the absorbance within **30 min** at **450 nm** with **≥ 590 nm** as reference wavelength.		

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