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IGFBP-6 ELISA

Enzyme Immunoassay for the Determination of

human Insulin-like Growth Factor Binding Protein 6 (IGFBP-6)

Concentrations

Not for Use in Diagnostic Procedures.

Strorage at	Kit size
2-8°C	96 wells

Cat.-No. E112

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IGFBP-6 ELISA E112	96 Determinations
RUO	n/a
Principle of the test	Enzyme-linked Immunoassay
Duration (incubation period)	3.0 h
Antibodies	specific, polyclonal rabbit anti-human IGFBP-6 antibodies
Cross reactivity with IGFBP-2 /-5 and IGF-I/-II	< ∅ 2 %
Buffer	Ready for use and 20fold concentrate
Standard	5 single standards: 0.1 – 10 µg/L, recombinant human IGFBP-6
Assay Range	0.026 – 500 ng/mL
Control	2 control sera, freeze-dried
Sample	human serum / plasma
Required sample volume	10 µL
Sample dilution	1:51
Analytical sensitivity	0.026 µg/L
Intra- / Interassay Variance	<10 %

1 INTENDED USE

Measurement of human IGFBP-6 in human serum / plasma and other body fluids for research use only. Not for use in diagnostic procedures.

2 INTRODUCTION

The Insulin-like Growth Factor 6 (IGFBP-6) is part of the IGF-System which consists of several binding proteins (1-6) and IGF-I and –II. The Insulin-like Growth Factors are involved in the control of human growth and regulated themselves by their binding proteins. The IGFBPs show high amino acid sequence homology, they have conserved N- and C-terminal domains which are involved in IGF-binding. In contrast the central linker region is highly variable. This region is not directly involved in IGF-binding but influences stability and localisation of the IGFBP / IGF complex and can be modified by glycosylation and phosphorylation.

IGFBP-6 a protein of 213 amino acids and about 34 kDa is somewhat special because of only three disulphide bridges in the C-terminal domain, resulting in a significantly higher affinity (50fold) for IGF-II than IGF-I. Further IGFBP-6 can be O-glycosylated and is cleaved by cathepsin-D-like acid protease, neutral serine protease as well as MMP-2/-7/-9/-12.

Main proposed function of IGFBP-6 is the regulation of the biological availability of IGF-II and thereby it influences cell proliferation, differentiation, migration and survival. Further IGF-independent as well as intra- and nuclear actions of IGFBP-6 are discussed. IGF-independent actions might be transmitted by prohibitin-2 as a potential cell surface receptor. Physiologically IGFBP-6 might be involved in senescence, angiogenesis and cancer. But a clear clinical indication for measurement of plasma IGFBP-6 needs to be determined (Bach, 2015).

3 PRINCIPLE

This ELISA assay is based on polyclonal antibodies and recombinant IGFBP-6, expressed in eukaryotic cells. This ELISA for IGFBP-6, E112 is a so-called Sandwich-Assay. It utilizes two specific antibodies of high affinity. First the IGFBP-6 in the sample binds to the immobilized antibody on the microtiter plate. In a two-step sequence, the biotin-conjugated anti-IGFBP-6-Antibody and the streptavidin-peroxidase are bound.

Subsequently, the peroxidase catalyzes an enzymatic reaction resulting in a blue coloration. The intensity of the blue color depends on the analyte content of the sample. The reaction is stopped by the addition of stop solution and color intensity (yellow) is quantified by measuring the absorption.

This IGFBP-6 ELISA, cat.-no. E112 allows secure and reproducible measurement of IGFBP-6 in human body fluids. In a preliminary study IGFBP-6 was measured in serum of healthy blood donors and mean concentration of 204 ng/mL was detected (Range: 73-367, n=20).

4 WARNINGS AND PRECAUTIONS

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

This ELISA kit is suitable only for research use. 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 obviously damaged or microbial contaminated or spilled material.

Caution: This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens 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. Material Safety Data Sheet is available on request.

4.1 Human Serum

Following components contain human serum: **Control Serum KS1 and KS2**
Source human serum for the control sera 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 patient´s specimens should be treated as potentially infectious.

4.2 Reagents

Reagents A-E, AK, EK, VP, WP
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 Solution (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.

Stopping Solution (SL)
The Stopping 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.

4.3 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 samples as well as other body fluids and cell culture medium

The influences of anti-coagulants on IGFBP-6 measurements by ELISA E112 were investigated in corresponding EDTA and serum samples. One of 10 samples showed a significant reduction of IGFBP-6 concentration in EDTA/Aprotinin Plasma compared with serum of ~40%. On average recovery of serum IGFBP-6 in EDTA (n=10) and heparin (n=5) plasma was 102 and 98%, respectively. Additionally, recovery of recombinant IGFBP-6 was evaluated in several body fluids (breast milk, saliva, amniotic fluid, urine) as well as in RPMI cell culture medium was tested. The recovery was not impaired in any tested fluids except urine.

5.2 Specimen collection

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

Required sample volume: **10 µL**

5.3 Sample stability

In firmly closed sample vials:

- Storage at 20-25°C: max. 2 days
- Storage at -20° C: min. 1 year
- Freeze-thaw cycles: **max. 1** (sample dependent an increase in signal intensity was detected after more cycles)

Therefore it is recommended to keep sample refrigerated or frozen as soon as possible after separation of coagulated and corpuscular blood components and to avoid more than 1 freeze-thaw cycles.

5.4 Interference

Generally, hemolytic, icteric and lipemic samples should be avoided. Interference was tested by addition of triglycerides, haemoglobin or bilirubin to serum samples and measurement of IGFBP-6 recovery. At 100 mg/mL, 5 mg/mL and 200 µg/mL no significant (<30%) interference was detected.

5.5 Sample dilution

- Dilution: **1:51** with **Dilution Buffer VP**
- Pipette **500 µL Dilution Buffer VP** in PE-/PP-Tube (application of a multi-stepper is recommended in larger series); add **10 µL sample** (dilution 1:51). After mixing use 2 x 100 µL of this dilution in the assay.
- Attention: serum and plasma samples must be diluted at least 1:20 in **Dilution Buffer VP.**
- Depending on the expected IGFBP-6 values the samples can be diluted higher in **Dilution Buffer VP**.
- Sample stability after dilution of the sample: maximum 1 hour at 20-25°C.

6 MATERIALS

6.1 Materials provided

The reagents listed below are sufficient for 96 wells including the standard curve.

MTP	**Microtiter plate,** ready for use, coated with rabbit-anti-hIGFBP-6-antibody. Wells are separately	**(8x12) wells**
A-E	**Standards**, lyophilized, (recombinant human IGFBP-6), concentrations are given on vial labels and on quality certificate.	**5 x 750 µL**
KS1	**Control Serum 1,** lyophilised, (human serum), concentration is given on quality certificate in ng/mL.	**1 x 250 µL**
KS2	**Control Serum 2,** lyophilised, (human serum), concentration is given on quality certificate in ng/mL.	**1 x 250 µL**
AK	**Antibody Conjugate,** ready for use, contains rabbit biotinylated anti-hIGFBP-6 antibody**.**	**1 x 12 mL**
EK	**Enzyme Conjugate,** ready for use, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin.	**1 x 12 mL**
VP	**Dilution Buffer,** ready for use	**1 x 120 mL**
WP	**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**
SL	**Stopping Solution,** ready for use, 0.2 M sulphuric acid.	**1 x 12 mL**
-	**Sealing Tape,** for covering the **microtiter plate.**	**3 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 **WP (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** standards **A - E** and Control Sera **KS1** and **KS2** 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 **WP** 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 Standards **A – E** and Control **KS1 and KS2** are reconstituted with the Dilution Buffer **VP**. 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 **KS1** and **KS2** with the Dilution Buffer **VP** in the same ratio (1:51) as the sample.
The required volume of Washing Buffer **WP** is prepared by 1:20 dilution of the provided 20 fold concentrate with Aqua dest.

Assay Procedure

When performing the assay, Blank, Standards **A-E**, Controls **KS1 and KS2** 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 **AK** and the Enzyme Conjugate **EK** as well as the succeeding Substrate Solution **S** should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution **SL** should be added to the plate in the same order as Substrate Solution **S**. All determinations (Blank, Standards **A-E**, Control **KS1** and **KS2** 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 Solution **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 approx**. 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 **WP** 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.

Manual washing should be used. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. Decant contents into a biohazard bin and then blot plate on absorbent tissue. Wash the plate by adding 300 µL Washing Buffer WP/well, then decant and blot on absorbent tissue. Repeat this step 4 more times for total of 5 washes.

8 ASSAY PROCEDURE

Preparation of reagents		Reconstitution:	Dilution
A-E	**Standards**	in **750 µL** Dilution Buffer **VP**	-
KS1	**Control Serum 1**	in **250 µL** Dilution Buffer **VP**	**1:51** with Dilution Buffer **VP**
KS2	**Control Serum 2**	in **250 µL** Dilution Buffer **VP**	**1:51** with Dilution Buffer **VP**
WP	**Washing Buffer**	-	**1:20** with **Aqua dest.**

Dilute samples 1:51 in Dilution Buffer VP, mix immediately, incubate max. 1h.
Use **100 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
100 µL	Dilution Buffer **VP** (Blank)	A1/A2
100 µL	Standard **A** (**0.1 ng/mL**)	B1/B2
100 µL	Standard **B (0.5 ng/mL)**	C1/C2
100 µL	Standard **C (1 ng/mL)**	D1/D2
100 µL	Standard **D** (**5 ng/mL)**	E1/E2
100 µL	Standard **E (10 ng/mL)**	F1/F2
100 µL	Control Serum **KS1** (1:51 diluted)	G1/G2
100 µL	Control Serum **KS2** (1:51 diluted)	H1/H2
100 µL	Sample (1:51 diluted)	in the rest of the wells according the requirements
Cover the wells with the sealing tape.		
Sample-Incubation: 1 h (+/- 5 Minutes) **at 20-25°C, 350 rpm**		
5x 300 µL	Decant the contents of the wells and **wash** 5 x with 300 µL each Washing Buffer **WP/ well**	In each well
100 µL	Antibody Conjugate **AK**	In each well
Cover the wells with the sealing tape.		
Incubation: 1 h (+/- 5 Minutes) **at 20-25°C, 350 rpm**		
5x 300 µL	Decant the contents of the wells and **wash** 5 x with 300 µL each Washing Buffer **WP/ well**	In each well
100 µL	Enzyme Conjugate **EK**	In each well
Cover the wells with the sealing tape.		
Incubation: 30 Minutes (+/- 5 Minutes) **at 20-25°C, 350 rpm**		
5x 300 µL	Decant the contents of the wells and **wash** 5 x with 300 µL each Washing Buffer **WP/ well**	In each well
100 µL	Substrate Solution **S**	In each well
Incubation: 30 Minutes (+/- 2 Minutes) **in the Dark at 20-25°C**		
100 µL	Stopping Solution **SL**	In each well
Measure the absorbance within 30 min at **450 nm** with ≥ 590 nm as reference wavelength.		

9 QUALITY CONTROL

Good laboratory practice requires that controls are included in each assay. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The test results are only valid, if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards/laws.

9.1 Quality criteria

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 standard E should be above 1.00. Samples, which yield higher absorbance values than **Standard E**, should be re-tested with a higher dilution.

Standard A (0.1 ng/mL) should be within +/-25% of its nominal value and ≤ 25% CV.
Standards B – E (0.5-10 ng/mL) should be within +/-20% of their nominal values and ≤ 20% CV.
Controls KS1 and KS2 should be ≤ 20% CV and be within the specified range.

The assay is valid when both control sera KS1 and KS2 as well as 5/6 standards met the acceptance criteria specified.

10 EVALUATION OF RESULTS

10.1 Establishing of the standard curve

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 and standards

3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.

4) 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 **four parametric logistic (4-PL) curve fit** should be used for recalculation of IGFBP-6 concentrations.

5) The IGFBP-6 concentration in ng/mL of the samples and controls KS1 and KS2 can be calculated by **multiplication** with the **dilution factor of 1:51.**

10.2 Example of a typical standard 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	0.1	0.5	1	5	10
OD **(450-620 nm)**	0.06	0.08	0.26	0.39	1.52	2.48

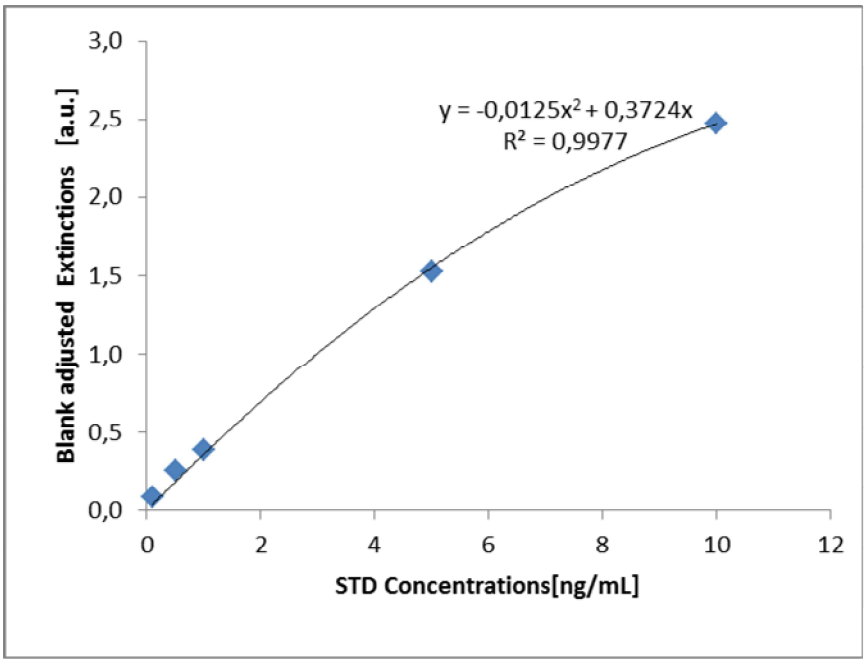

Figure 1 Exemplary Standard Curve

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

10.3 Exemplary calculation of IGFBP-6 concentrations

Sample dilution: 1:51

Measured extinction of your sample: 1.495
Measured extinction of the blank 0.055

Your measurement program will calculate the IGFBP-6 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.

In this exemplary case the following equation is solved by the program to calculate the IGFBP-6 concentration in the sample:

$$y = -0.0125x^2 + 0{,}3724x$$

$$1.44 = x$$

If the dilution factor (**1:51**) is taken into account the IGFBP-6 concentration of the undiluted sample is

$$1.44 \times 51 = 73.44 \text{ ng/mL}$$

10.4 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.

11 LIMITATIONS OF PROCEDURE / FACTORS INFLUENCING IGFBP-6

IGFBP-6 levels increase with age and are higher in men than in women, serum levels decrease during pregnancy and increase in renal failure. In vitro IGFBP-6 expression is influenced by cAMP, IGFs, retinoic acid, Vitamin D, p53 and glucocortioids depending on the investigated cell model. TNF-β and β-catenin inhibit IGFBP-6 promoter activity (Bach, 2015).

The Mediagnost IGFBP-6 ELISA, E112 is based on polyclonal antibodies from rabbit. 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.

12 REFERENCE VALUES

IGFBP-6 was measured in blood samples of healthy blood donors. Measured mean is 204 ng/mL (Range: 73 – 367).

13 PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

Sensitivity was assessed by measuring the signal of the blank at least 8 times within one test and calculating the theoretical concentration of 2SD of the mean signal measured. The analytical sensitivity of the Mediagnost E112 is on average 0.026 ng/mL, measured in 4 independent tests (Range 0.004 to 0.029 ng/mL).

Based on the minimal required dilution (1:20) and lowest calibration standard (0.1 ng/mL) the lower limit of quantification is 2 ng/mL in the undiluted sample.

13.2 Specificity

Influence of IGFs and IGFBP-2 to -5 on IGFBP-6 measurement was evaluated by direct measurement of potentially cross reactive substances in dilution buffer as well as on IGFBP-6 recovery in standard preparations.

Table 1 Specificity. Cross reactivity of IGF and IGFBPs in IGFBP-6 measurement. The recombinant proteins were diluted in assay buffer to the indicated concentrations and applied as sample. Further STD E was reconstituted in the enriched buffer and the IGFBP-6 concentration was measured. Here the the relative cross reactivity and recovery are shown at two concentrations of the IGFBPs and IGFs.

	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGF-I	IGF-II
[ng/mL]	250	600	250	250	500	500
Cross reactivity in VP [%]	0,0	0,1	0,0	0,0	0,0	0,0
Recovery of STD E [%]	0	5	-3	-7	-5	-17
[ng/mL]	50	300	50	50	100	100
Cross reactivity in VP [%]	0,05	0,08	0,05	0,02	0,00	0,03
Recovery of STD E [%]	-3	2	-4	-9	-10	-9

13.3 Precision

Intra-Assay Variance

IGFBP-6 content of four samples was measured at least 12 times in the same assay. The results are shown in Table 2. The measured coefficient of variation (CV) is 1.1% on average.

Table 2 Intra-assay variability. The IGFBP-6 concentration was measured 12 or more times in an assay and the variability was calculated as coefficient of variation (CV).

CV [%]	Sample 1	Sample 2	Sample 3	Sample 4
Test 1	1,63	2,24	1,91	4,15
Test 2	1,65	1,99	2,45	2,49
Test 3	1,10	2,66	3,89	1,65
Test 4	4,57	4,06	4,09	1,48
Test 5	1,50	2,89	1,84	2,20
Test 6	1,56	2,43	3,18	1,87

Inter-Assay

IGFBP-6 content of 12 serum samples was measured in 11 or more independent assays. Exemplary results are shown in Table 3.

Table 3 Inter-Assay variability. Samples were diluted and IGFBP-6 content measured in independent assays.

Sample	1	2	3	4	5	6	7	8	9	10	11	12
Mean [ng/mL]	127	203	187	177	191	109	264	174	73	190	74	151
CV [%]	**7**	**11**	**4**	**8**	**8**	**9**	**4**	**4**	**7**	**5**	**6**	**7**
[n]	18	13	13	13	18	21	16	11	13	16	11	11

13.4 Linearity

Linearity of the Mediagnost E112 test system was tested by dilution (1:5 up to 1:640) of native sera with different IGFBP-6 contents (Sample 1-3). A comparison of expected and measured concentrations is shown in Figure 2. It becomes apparent that a dilution of 1:10 is not sufficient to allow linearity in all samples. These results were confirmed according to NCCLS/CLSI EP6-A 2003 standard by statistical analysis. A **minimal dilution of ≥ 1:20** is required for linearity. The linearity of sample dilution is acceptable within the standard curve range.

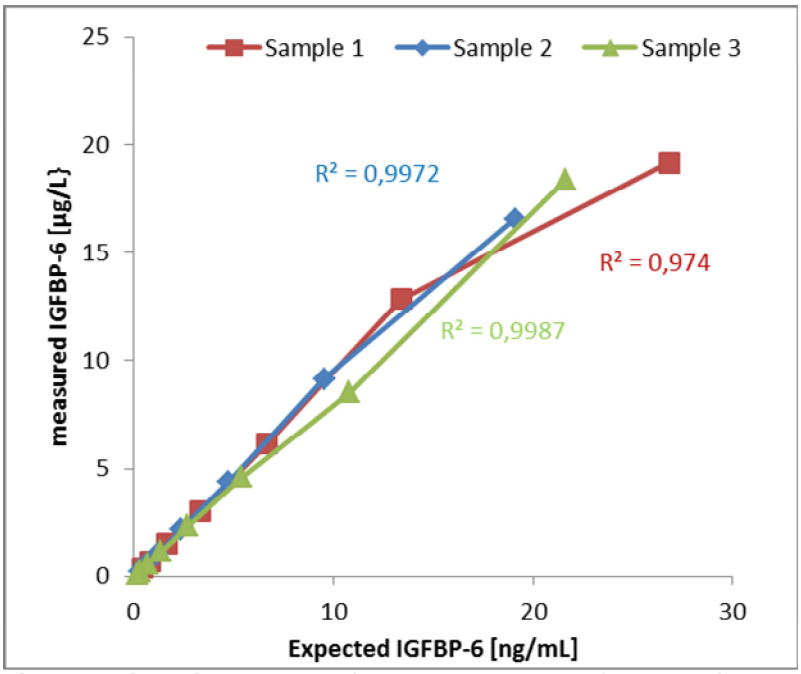

Figure 2 Linearity, measured IGFBP-6 concentrations are shown on y-axis in comparison to the theroretically expected concentration. Linearity up to a concentration of 0.5 ng/mL is given, provided the dilution is1:20.

13.5 Recovery

Recombinant IGFBP-6 was added in different amounts to human serum. The IGFBP-6 content of the so enriched samples was measured and recovery in comparison to enriched buffer calculated. Results are shown in Table 4.

Table 4 Recovery of recombinant IGFPB-6 in human serum. Recombinant IGFBP-6 was added in two different concentrations to three human serum samples. The IGFBP-6 content of enriched and non-enriched samples was measured and the relative recovery of added IGFBP-6 calculated.

rec. IGFBP-6 [ng/mL]	Recovery as relative expected value [%]		
	Sample 1	Sample 2	Sample 3
272	94.98	94.50	95.59
4.573	101.73	102.36	103.81

13.6 Interference

Interference of haemoglobin, bilirubin and triglycerides was tested by adding different amounts of these substances to human serum containing IGFBP-6. For comparison the same amount of buffer without any substance was also added to the serum. Table 5 demonstrates that neither haemoglobin, bilirubin nor triglycerides exert significant influence on the measurement of IGFBP-6 in human serum.

Table 5 Interference of physiologic substances on IGFBP-6 measurement. Recovery of IGFBP-6 after adding potentially interfering substances.

[%]	Triglyceride	Bilirubin	Hämoglobin
	100 mg/mL	200 µg/mL	5 mg/mL
Sample 1	99	96	68
Sample 2	96	100	74
Sample 3	108	107	81

13.7 Traceability / Assay Calibration

Recombinant IGFBP-6 produced by mammalian, eukaryotic cells and of >98% purity (SDS-PAGE, Silverstain) is used as standard within the assay. International Standard or Reference preparations are not available. Therefore, traceability is realized by Mediagnost internal serum panels.

13.8 Standard Curve

Uncertainty of the standards was measured as precision of recalculated standard concentration. Relative deviation corresponds to uncertainty. Variability of the recalculated STD concentrations was less than 20% for all standards, as well as %RE (Range -18.1 to 12.6%). Results are shown in Table 6.
The statistically significant differentiation of the signal intensity of STD A compared with the blank or STD B was also shown by Welch´s t-test in three independent experiments (n = 8, p <0.01).

Table 6 Uncertainty of the standards. Comparison of the recalculated IGFBP-6 concentrations of the individual standard points of six independent tests is shown. The calculation was based on the 2nd degree polynomic function for curve fitting.

	STD A	**STD B**	**STD C**	**STD D**	**STD E**
[ng/mL]	0.1	0.5	1	5	10
Test 1	0.10	0.50	1.01	5.00	10.00
Test 2	0.10	0.46	0.98	5.02	9.99
Test 3	0.09	0.59	0.97	4.99	10.01
Test 4	0.13	0.51	1.02	4.99	10.00
Test 5	0.11	0.48	0.99	5.01	10.01
Test 6	0.11	0.51	0.99	5.00	10.00
Mean [ng/mL]	0.11	0.51	0.99	5.00	0.11
CV [%]	12	9	2	0	0

13.9 Cross reactions with animal samples

Several commercially available animal sera have been used as samples in this IGFBP-6 ELISA cat.-no. E112 in a dilution of 1:51 no significant signal (< STD A) was detected. Thus, this assay cannot be used for IGFBP-6 measurement in serum samples of cat, cattle, chicken, dog, donkey, goat, guinea pig, horse, mouse, pig, rabbit, rat and sheep.

14 Literature

Taferner A, Micutkova L, Hermann M, Pidder JD, Pircher H Purification and characterization of native human insulin-like growth factor binding protein-6 J Cell Commun. Signal (2011) 5:277-289.

Bach LA Recent insight into the actions of IGFBP-6. J Cell Commun Signal (2015) 9(2):189-200.

International Assay Description

A-E	**STD**	**Rec in 750 µL BUF VP**	**-**
KS1	**Control**	**Rec in 250 µL BUF VP**	**1:51 DILU BUF VP**
KS2	**Control**	**Rec in 250 µL BUF VP**	**1:51 DILU BUF VP**
WP	**WASHBUF 20x**	**-**	**1:20 DILU A. dest.**
-	**SPE**		**1:51 DILU BUF VP**
-	**°C 20-25 °C ; max. 1 h; ↔ max 350 rpm**		

100 µL	**BUF VP**	A1/A2
100 µL	**STD A (0.1 ng/ml)**	B1/B2
100 µL	**STD B (0.5 ng/ml)**	C1/C2
100 µL	**STD C (1 ng/ml)**	D1/D2
100 µL	**STD D (5 ng/ml)**	E1/E2
100 µL	**STD E (10 ng/ml)**	F1/F2
100 µL	**CONTROL KS 1 1:51 DILU BUF VP**	G1/G2
100 µL	**CONTROL KS 2 1:51 DILU BUF VP**	H1/H2
100 µL	**SPE 1:51 DILU BUF VP**	
	TAPE	
	1 h °C 20-25 ↔ 350 rpm	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**Ab AK**	
	TAPE	
	1 h °C 20-25 ↔ 350 rpm	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**CONJ EK**	
	TAPE	
	0.5 h °C 20-25 ↔ 350 rpm	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**SUBST TMB S**	
	0.5 h °C 20-25 ▷	
	H_2SO_4 SL	
	MEASURE	