IGFBP-3 LIA

Ligand-binding Immunoassay
for Quantitative Determination of

Functional
Insulin-Like Growth-Factor Binding Protein-3

English

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

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Symbols / Symbole

according to DIN EN 980 and EDMA recommendations Standard News 6 2001

		Expiry date / Verfallsdatum / Date de péremption / Data di scadenza /Fecha de caducidad / Data de validade / Uiterste gebruiksdatum / Udløbsdato / Utgångsdatum / Termin Ważności / Lejárati idő / Doba exspirácie / Doba exspirace
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		Store at between / Lagerung bei zwischen / Conserver à entre / Conservare a tra / Conservar a temp. entre / Armazene a entre / Bewaar bij tussen / Opbevares mellem / Förvaras vid / Przechowywać w / Tároljuk ð ð között / Skladujte v rozsahu / Skladujte v rozmezí
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		Keep away from sunlight / Nicht dem Sonnenlicht aussetzen
		Incubation time / Inkubationszeit
°C		Incubate at / Inkubation bei
↔		Shaking / schütteln
MTP		Miktrotiter plate/Mikrotiterplatte
Rec in		Reconstitute in / Rekonstituieren in
SPE		Sample / Probe
LIGAND	LK	Ligand Conjugate / Ligandkonjugat
CONJ	EK	Enzyme Conjugate / Enzymkonjugat
DILU X	VP/PP	Dilute in Buffer X / Verdünnen in Puffer X
CAL X	A-E	Standard X / Standard X
Control	KS1/KS2	Control Serum / Kontrollserum
WASHBUF 20x	WP	Washing Buffer Concentrate / Waschpufferkonzentrat
WASHBUF		Washing Buffer / Waschpuffer
SUBST TMB	S	Substrate / Substrat
H_2SO_4	SL	Stop Solution / Stopplösung
TAPE		Cover Plate with sealing tape / Platte abkleben
MEASURE		Measure plate within 30 min at 450 nm (Referencefilter ≥590nm) / Ausmessung innerhalb von 30 min bei 450 nm (Referenzfilter ≥ 590 nm).

For in vitro use only!
For Research Use Only!
For professional use only!
CAUTION: Not for human or animal therapeutic or diagnostic use.
Read entire protocol before use!

TECHNICAL PROPERTIES AND APPLICATIONS

Mediagnost IGFBP-3 LIA E04A

- For research and professional use only!
- Quantitative determination of **functional bioactive (IGF-I binding) IGFBP-3: convenient "ELISA", no sample pretreatment**
- solely **functional bioactive IGF-I-binding IGFBP-3** is being quantified, proteolytic fragments without binding affinity to the **natural ligand IGF-I** are not measured
- **Inter-Assay variation** of max. **6.8%, Intra-Assay variation** of max. **5.6%**
- reliable, fast and simple: performance like a conventional ELISA
- **Total IGFBP-3** integrates the GH secretory state over days, with stable serum levels due to absence of circadian variation a single measurement is highly informative for diagnosis of GH deficiency or GH excess: **correspondent measurement of functional IGFBP-3** reflects the respective degree of fragmentation of IGFBP-3 and thus allows further interpretation of the results
- **Direct Correlation** to the quantitative results of the immune reactive total IGFBP-3 from the respective Mediagnost Kits (E03A, IGF-R10, IGF-R11). In retrospect, or, in simultaneous determinations out of the same sample dilution!
- Small sample requirement, thus ideal for pediatric patients

INTRODUCTION

MEASURING IGFBP-3

All currently existing IGFBP-3 immunoassays use the binding of specific anti-IGFBP-3 antibodies for signal generation and thus IGFBP-3 quantification. The failure of differentiation between complete IGFBP-3 molecules and their respective fragments (derived physiologically due to the different proteases activities) is unavoidable in this system. Because one molecule IGFBP-3 can be cleaved in several fragments often false high quantitative values are measured. Based on this methodology it is not possible to differentiate between high IGFBP-3 levels in fact, or, a high degree of fragmentation. The incidental attempts to use monoclonal antibodies with a binding region represented only by the intact IGFBP-3 molecule are indirect, imprecise and insufficient. The activities of all effective proteases, which have different sites of action and therefore generate different kind of fragments, are disregarded.

The Mediagnost IGFBP-3 LIA however enables to determine the **real functional and effective bioactive IGFBP-3**, functional in terms of binding ability of the mainly interesting natural ligand, namely IGF-I !

The new test principle (patent pending DE19719001) uses anti-IGFBP-3 antibodies immobilized on the microtitre plate and biotinylated IGF-I as ligand. The IGFBP-3 of the sample is bound to the microtitre plate, and only concomitant bound, by IGFBP-3, biotinylated IGF-I gives the specific signal of the test. Therefore only functional IGFBP-3 is quantificated. The patented test format ensures an easy and reliable performance, simply like a conventional ELISA kit. Advanced and time- as well as labor-intensive biochemical analysis (e.g., by size chromatography or Western Blots, etc.), which moreover only allows an estimation of concentrations, has become redundant.

TOTAL IGFBP-3

Insulin-like growth factors (IGF)-I and -II are bound to specific binding proteins (IGFBPs) in the circulation. To date, at least six binding proteins can be distinguished on the basis of their amino acid sequence. They are designated as IGFBP-1, IGFBP-2, ...IGPBP-6 (1). Lately the discovery of a new IGFBP-7 has been discussed (2). The predominating IGFBP in blood is IGFBP-3. In contrast to the other binding proteins, IGFBP-3 has the unique property to associate with an acid-labile non-binding subunit (ALS) after binding of either IGF-I or IGF-II (3-5). Most of the IGFBP-3 in plasma is present as the high molecular weight ternary complex, however, small amounts of free IGFBP-3 are also found (6, 7).

The development of specific immunoassays for IGFBP-3, those also recognizing the complete high molecular weight complex, provided new in-sights into its regulation (6-9). On the basis of these findings total serum IGFBP-3 has proved to be an additional useful test in the repertoire of diagnostic tools for evaluation of growth disorders (7, 8).

Patients with GH deficiency have subnormal total IGFBP-3 levels. In contrast, most of the small statured children with normal GH secretion have levels within the normal range (Figure 1).
The separation of these two groups is easy. A single measurement of the total IGFBP-3 concentration is sufficient for the diagnosis of GH deficiency with high accuracy (7, 18). In small statured children total IGFBP-3 levels rise to normal range within several days of GH administration and remain normal during continuous GH treatment (Figure 2). Therefore, total serum IGFBP-3 measurements are also suited for evaluating the potential of a patient to respond to GH and for GH therapy monitoring (19). In other patients of severe short stature, e.g. Ullrich-Turner syndrome or Silver-Russell syndrome, IGFBP-3 levels were found normal (8) reflecting normal GH secretion.
In normal tall children and adolescents without excessive GH secretion or in patients with Sotos syndrome, total IGFBP-3 levels are normal or slightly increased. In contrast, children with pituitary gigantism or adults with acromegaly have clearly elevated levels (Figure 3) (6, 15) that normalize on successful treatment. Therefore, total IGFBP-3 is also a useful parameter for the detection of excessive GH secretion and monitoring therapy efficacy. In precocious puberty, total IGFBP-3 levels are clearly increased by chronological age, whereas patients with premature thelarche have total IGFBP-3 levels in the upper normal range (15).
Several factors besides GH influence total IGFBP-3 levels: age including sexual development, nutrition, hypothyroidism, diabetes mellitus, liver function and kidney function. Total IGFBP-3 levels are decreased by malnutrition, although less than IGF-I, in hypothyroidism, in diabetes mellitus and in hepatic failure (6-8), but are obviously increased in chronic renal failure (6, 10, 11). Measurement over 24 hours revealed constant circadian levels (12, 13). For clinical practice, the most important regulatory factor is GH. Single total IGFBP-3 measurements correlate significantly with the logarithm of the integrated spontaneous GH secretion (8, 14). In patients with GH deficiency, total IGFBP-3 levels are subnormal and increase gradually to within the normal range after several days of GH administration (7, 8). The slow response to GH and constant circadian levels during chronic daily application of GH (13) suggest that IGFBP-3 reflects the GH secretory state over days.

FRAGMENTED IGFBP-3

By proteolytical cleavage of the ternary complex of IGFBP-3, the physiological storage of IGF-I in circulation, IGF-I am released and subsequently able to bind to its cellular receptor.
IGFBP-3 can be cleaved by several proteases: Plasmin; PSA; MMPs; CathepsinD, Thrombin, gamma NGF.
Cleavage results not only in free IGF-I but also in different IGFBP-3 fragments. Dependent on the active protease, 22 cleavage sites are known, mostly located in the variable and the N-terminal region but some also in the C-terminal part. Fragments of about 30, 20 and 15 kDa can be generated by proteolysis. Their existence was proven by western ligand blotting. The

corresponding immunoblot demonstrated that not all fragments are able to bind IGF-I any more. So cleavage of IGFBP-3 results in at least partial loss of IGF-I affinity (20).

IGFBP-3 proteolysis can be found in a number of body fluids, like synovial fluid, amnion fluid, seminal fluid, interstitial fluid, peritoneal fluid, lymph and serum, of course. In all these body fluids different proteases can be activated resulting in a different fragmentation pattern of IGFBP-3 of different relevance for physiology (21).

Beside pregnancy, where nearly all serum IGFBP-3 is fragmented, several pathological conditions are known where the fragmentation level of IGFBP-3 is changed.

In certain previous studies for determining the degree of fragmentation of IGFBP-3 more sophisticated and laborious biochemical methods were involved. In these studies, in healthy individuals around 25 – 30% of serum total IGFBP-3 was found to be proteolyzed.

Assay Characteristics

It utilizes specific and high affinity antibodies for IGFBP-3, the antibodies are immobilized on the microtitre plate. The ligand, biotinylated IGF-I is pre-dispensed in excess into the needed wells. The sample is diluted outside within a special dilution buffer (Sample Buffer PP); all naturally bound IGFs are thereby released from their binding proteins. By adding an aliquot of such diluted sample with free IGFBP-3 to the ligand containing wells, the biotinylated IGF-I occupies all existing specific binding sites of the IGFBP-3. All IGFBP-3 molecules are bound afterwards to the microtitre plate, however by using a Strepavidin-Peroxydase-(POD)-Conjugate only the complexes of IGFBP-3/biotinylated IGF-I are involved in signal generation (in the closing POD-substrate reaction), thus, only functional IGFBP-3 is being quantified !

The standards of the LIA E04A are made of **native and functional human IGFBP-3** in concentrations of **0.4, 2, 6, 15 and 30 ng/ml**.

The **analytical sensitivity** of the LIA E04A has been determined with **0.18 ng/ml** (2 SD of zero standard in 16fold determination).

The Mediagnost IGFBP-3 LIA E04A is over a very wide range dilution true. The **Linearity of the dilution of sera** is excellent (s. Table 1).

Table 1: The linearity of the sample dilution

Dilution:	**Sample 1** (recalculated, ng/ml)
1:100	2817
1:200	2962
1:400	3094
1:600	3055
1:800	3229
1:1000	2951
1:1200	2908
1:1600	2989
AV / SD / VC%	3001 / 125 /4.2

AV = Average Value, **SD** = Standard deviation **VC** = Variation Coefficient%

The **Inter**- and **Intra-Assay** variation coefficients were found lower than **6.8% and 5.6%**. Exemplary determinations are shown in table 2 and table 3.

Table 2: Inter-Assay-Variation (n=8)

	Mean Value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	**1051**	**30**	**3.0**
Sample 2	**1891**	**129**	**6.8**
Sample 3	**2417**	**158**	**6.5**

Table 3: Intra-Assay-Variation (n=16)

	Mean Value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	**1061**	**26**	**2,5**
Sample 2	**1571**	**85**	**5.4**
Sample 3	**2660**	**148**	**5.6**

FRAGMENTED IGFBP-3

The results determined with the human IGFBP-3 LIA E04A are only for research use!
Due to the new developed test system there are at present no concrete and specific clinical data available. Instructive might be the comparison of total IGFBP-3 values versus functional IGFBP-3 values in different subsets of samples.

Figure 4 shows results of comparative determinations of sera of healthy blood donors with the functional IGFBP-3 LIA E04A for functional IGFBP-3 and for total immunoreactive IGFBP-3 with Mediagnost kits, respectively. Functional IGFBP-3 values were found consistently lower on average compared with the corresponding total IGFBP-3.

Thereby a slight gender difference was obvious. Total IGFBP-3 serum concentrations of males were found lower than those of females. Functional IGFBP-3 concentrations of females and males however were found with nearly equal absolute values. Functional IGFBP-3 serum concentrations were found on average 25 % lower (Tab. 4; Fig 4).

Tab. 4: Mean total and functional IGFBP-3 values of sera of 103 female and 109 male healthy blood donors (IGFBP-3 concentrations in ng/ml). Average age was 42 years in each subset.

	Total IGFBP-3; min/max (ng/ml)	**Functional IGFBP-3; min/max (ng/ml)**	**Functional IGFBP-3 (% of total)**
Females	**3568; 1752/5752**	**2506; 1102/4346**	**70,2**
Males	**2752; 1258/4726**	**2332; 1337/4304**	**84,8**
overall	**3160**	**2419**	**77,5**

In a subset of pathological sera of donors suffering from different diseases substantially lower functional IGFBP-3 concentrations were obvious (Fig. 5). Further and more detailed studies however are necessary to reveal the underlying mechanisms and, from this to develop new diagnostic insights and processes.

WARNINGS AND PRECAUTIONS

The functional **IGFBP-3 LIA, E04A** is for in-vitro 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.

The shelf life of the components after opening is not affected, if used appropriately.
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 Seren KS1 und KS2**
The source of human serum was 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) antibodies. 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 Sulphuric 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, 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, 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.

REAGENTS PROVIDED

1)	MTP	**Microtiter plate**, ready for use, Microtiter plate with 96 wells, divided up in 12 strips à 8 wells (separately breakable). Coated with an antibody against human IGFBP-3.
2)	CAL	**Standards A-E, 1 ml**, lyophilised, contain native human functional IGFBP-3. Standard values are between 0.4 - 30 ng/ml (0.4, 2, 6, 15 and 30 ng/ml) functional IGFBP-3. Standards are to be reconstituted with 1 ml Sample Buffer PP each. Use 50 µl per well in the assay.
3)	DILU	**Dilution Buffer VP, 30 ml**, ready for use, please use **for the dilution of the Ligand Conjugate LK and the Enzyme Conjugate EK**
4)	DILU	**Sample Buffer PP, 120 ml**, ready for use, green colored, please use for **reconstitution of Standards** and **Control** and for **dilution of the Samples and Control.**
5)	Control	**Control Seren KS1 and KS2, 250 µl**, lyophilised, contain human Serum and should be reconstituted in 250 µl Sample Buffer PP. The functional IGFBP-3 target values and the respective ranges are given on the vial labels. The dilutions should be according to the dilution of the respected samples. Use 50 µl per well in the assay.
6)	Ligand	**Ligand Conjugate LK, 100 µl**, 101-fold Concentrate, contains biotinylated recombinant human IGF-I. Before use dilute 1:101 with VP. Use 50 µl per well in the assay.
7)	CONJ	**Enzyme Conjugate EK, 140 µl**, 101-fold Concentrate, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin. Before use dilute 1:101 with VP. Use 100 µl per well in the assay.
8)	WASHBUF 20x	**Washing Buffer (WP), 50 ml**, 20 X concentrated solution. Washing buffer has to be diluted 1:20 with A.dest. or demineralised water before use (e.g. add the complete contents of the flask 50 ml into graduated flask and fill with A.dest to 1000 ml). Attention: After dilution, the Washing Buffer is only 4 weeks stable, please dilute only according to requirements.
9)	SUBST	**Substrate (S), 12 ml**, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbencidine.
10)	H_2SO_4	**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 and multichannel pipettes with disposable plastic tips
Distilled or deionized water for dilution of the Washing Buffer (WP)
Vortex-mixer
Microtiter plate shaker (350 rpm)
Microtiter plate washer (recommended)
Micro plate reader ("ELISA-Reader") with filter for 450 and ≥590 nm
Polyethylen PE/Polypropylen PP tubes for dilution of samples

TECHNICAL NOTES

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.

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

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

The incubation steps should be performed at mean rotation frequency of a particularly suitable microtitre plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must become 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.

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 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 microtitre 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 dynamicly swinging out the microtitre 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

Standards and Control
The Standards **A – E** and **Control Sera KS1 and KS2** are reconstituted with the **Sample Buffer PP** provided in the Kit. It is recommended to keep the reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.
Reconstituted components (Standards (A – E) and Control Sera (KS1/KS2)) 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. Freezing extends the expiry at least 2 months. Avoid repeated freeze-thaw cycles. In case you plan to perform multiple independent IGFBP-3 determinations over a longer period with one kit, you should aliquot the components prior to freezing into suitable smaller volumes. This is strongly recommended.

Ligand and Enzyme Conjugate
Use the Dilution Buffer **VP** for the dilution of Ligand Conjugate **LK** and Enzyme Conjugate **EK** concentrates. The diluted solutions are only limited stable at 2-8°C.

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 H_2O_2-Tetramethylbencidine, is photosensitive – store and incubate in the dark.

Microtiterplate
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.

SPECIMEN COLLECTION, PREPARATION AND STORAGE

Serum samples as well as Heparin- and EDTA-Plasma samples are suited. Possible dilution of the sample by the anticoagulant must be considered. Citrate-Plasma samples are not recommended, values are reduced. A special external sample preparation prior to assay is not required.

Slight Hemolysis of the samples obviously doesn´t disturb the determination. An external sample preparation prior to assay is not required (see below).Samples should be handled as recommended in general: collected and refrigerated as fast as possible. In case there will be a longer period (>24 hours) between the sample withdrawal and determination, store the undiluted samples frozen at –20°C or below in tightly closable plastic tubes. Avoid on principal repeated freeze-thaw cycles of serum/plasma (if required, please sub-aliquot) although functional IGFBP-3 levels were found to be unaffected by a few cycles, (3x) in our experiments. The high sensitivity of the assay allows the functional IGFBP-3 measurement in small sample volumes.

In most determinations (e.g., Serum- or Plasma samples and no extreme values expected) the dilution of **1:505 with Sample Buffer PP is suitable**, thus the respective covered **assay range is 0.2 to 15.15 mg/l.** Where required, depending on the expected IGFBP-3-values, the dilution with **Sample Buffer PP** can be higher or lower. The IGFBP-3 concentrations maybe completely different in body fluids of human origin other than serum or in cell culture supernatants.

Suggestion for dilution protocol:

Pipette **1 ml Sample Buffer PP** (green colored) in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add **10 µl Serum**- or **Plasma** (dilution 1:101). Add 400 µl Sample Buffer **PP** in an other PE-/PP-tube and 100 µl of the thoroughly mixed first dilution (dilution 1:5). After mixing use **50 µl** of this 1:505 diluted solution within 1 hour **per determination** in the assay (pipetting control = blue coloring of the solution in the wells).

ASSAY PROCEDURE

NOTES: All determinations (Standards, Control Serum 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 pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, the **Enzyme Conjugate**, **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.

1) Please dilute the Ligand Conjugate LK 1:101 with **Dilution Buffer VP** (for use of the entire Microtitre Plate you may need 4.8 ml, thus you may dilute 60 µl of concentrated **LK** with 6 ml of buffer **VP**).
2) Add in every needed well **50 µl** of the already 1:101 diluted **Ligand Conjugate LK**
3) Add **50 µl Sample Buffer PP** in positions A1/2 (=blank wells!)
4) Pipette in positions B1/2 **50 µl** each **Standard A (0.4ng/ml)**,
   pipette in positions C1/2 **50 µl** each **Standard B (2 ng/ml)**,
   pipette in positions D1/2 **50 µl** each **Standard C (6 ng/ml)**,
   pipette in positions E1/2 **50 µl** each **Standard D (15 ng/ml)**,
   pipette in positions F1/2 **50 µl** each **Standard E (30 ng/ml)**.
   To control the correct test accomplishment **50 µl** of the 1:505 (or in respective dilution rate of the sample) in Sample Buffer **PP** diluted **Control Sera KS1** and **KS2** can be pipetted in positions G1/2 and H1/H2.

Pipette **50 µl each** of the **diluted sample** (generally 1:505 diluted in Sample Buffer **PP**) in the rest of the wells, according to requirements. Please mix the dilutions immediately after sample addition and use within 60 minutes.

5) Cover the wells with the sealing tape and incubate the plate for **2 hours** at **room temperature** (shake at 350 rpm).
6) After incubation aspirate the contents of the wells and wash the wells **5 times** with **300 µl Washing Buffer WP**.
7) Following the last washing step, pipette **100 µl** of the **1:101 Enzyme Conjugate EK** in each well (for use of the entire Microtitre Plate you may need 9.6 ml, thus you may dilute 120 µl of concentrated **EK** with 12 ml of buffer **VP**).
8) Cover the wells with the sealing tape and incubate **1 hour** at **room temperature** (shake at 350 rpm).
9) After incubation wash the wells **5 times** with **Washing Buffer WP** as described in step 6
10) Pipette **100 µl of the TMB-Substrate solution S** in each well.
11) Incubate the plate for **30 Minutes in the dark at** room **temperature**.
12) After incubation pipette **100 µl Stop Solution SL** in each well.
13) Measure the absorbance **within 30 minutes at 450 nm (Reference filter ≥590 nm)**.

QUALITY CONTROL

Good laboratory practice requires that controls be run with each calibration curve. 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. All standards and kit controls must be found within the acceptable ranges as stated on the QC Certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

CALCULATION OF RESULTS

Establishing the Standard Curve

For the evaluation of the assay it is preconditioned that the absorbance values of the blank should be 0.25, these of standard E should be above 1.0.

Samples, which yield higher absorbance values than Standard E, are beyond the standard curve, for reliable determinations these samples should be tested anew with a higher dilution.

The standards provided contain the following concentrations of functional IGFBP-3:

Standard	**A**	**B**	**C**	**D**	**E**
ng/ml	0.4	2	6	15	30

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 values.
3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
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 functional IGFBP-3 concentration in ng/ml of the samples can be calculated by multiplication with the respective dilution factor, Division by 1000 converts the values in µg/ml or, equal mg/Litre (Example: a measured value was 6 ng/ml, Sample was 1:505 diluted: 6 x 505= 3030 ng/ml, or 3,03 µg/ml or 3,03 mg/L, according the requested unit).

LIMITATIONS

IGFBP-3 levels are strongly dependent on GH secretion. However, a number of factors influence its plasma concentration and should be taken into account for appropriate interpretation. Plasma levels decrease during fasting (more than 1 day), in malnutrition, malabsorption, cachexia, impaired hepatic function, hypothyroidism, and diabetes mellitus. They may also be decreased in chronic inflammatory disease and malignancy. Levels are increased in states of impaired renal function and precocious puberty. In clinical situations with hyperprolactinemia or in patients with craniopharyngeoma, normal levels may be observed despite GH deficiency. In certain physiological (e.g. pregnancy) and pathological states, IGFBP-3 may be degraded to smaller molecular size compounds (16, 17) by specific proteases which affect IGFBP patterns.

EXEMPLARY VALUES

Exemplary values of fragmented IGFBP-3 in relation to total IGFBP-3 of healthy blood donors are shown in figure 1. It is demonstrated that about 25% of the IGFBP-3 is fragmented and thus could be detected by biotinylated IGF-I. In table 5 distribution of IGFBP-3 in healthy blood donors of different age and sex are shown.

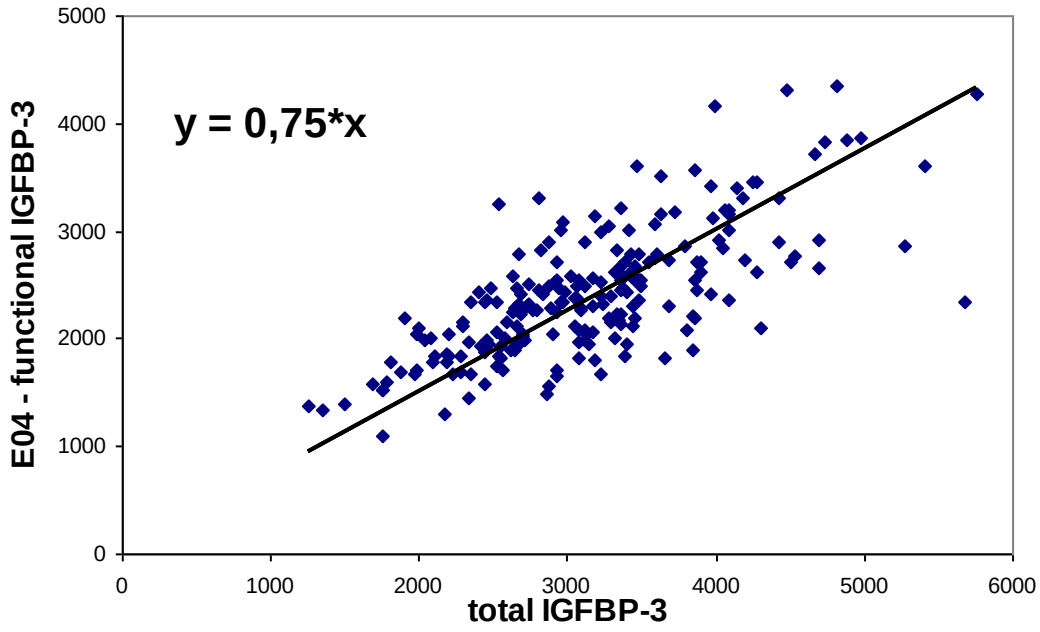

Figure 1: Plot of functional IGFBP-3 values against total IGFBP-3 values of sera from 212 healthy blood donors (IGFBP-3 concentrations in ng/ml).

Tab. 5: Serum levels of total immunoreactive IGFBP-3 in healthy subjects at various ages. Individuals between 7 and 17 years of age were classified according to gender, as the pubertal peak occurs almost 2 years earlier in girls than in boys.

Altersgruppe / Age group		**Percentiles / Perzentilen**													
		0.1	1	**5**	10	**20**	30	**40**	50	**60**	70	**80**	90	**95**	99
0-1 week		**0.25**	0.33	**0.42**	0.48	**0.57**	0.64	**0.70**	0.77	**0.85**	0.93	**1.05**	1.23	**1.41**	1.81
1-4 weeks		**0.49**	0.62	**0.77**	0.86	**0.99**	1.10	**1.19**	1.29	**1.40**	1.52	**1.68**	1.93	**2.16**	2.68
1-3 months		**0.55**	0.70	**0.87**	0.98	**1.13**	1.25	**1.36**	1.48	**1.61**	1.75	**1.94**	2.23	**2.52**	3.14
3-6 months		**0.64**	0.80	**0.98**	1.10	**1.25**	1.38	**1.49**	1.61	**1.74**	1.88	**2.07**	2.37	**2.65**	3.24
6-12 months		**0.71**	0.88	**1.07**	1.19	**1.35**	1.48	**1.60**	1.72	**1.85**	2.00	**2.19**	2.49	**2.76**	3.36
1-3 years		**1.02**	1.21	**1.41**	1.53	**1.69**	1.82	**1.94**	2.05	**2.17**	2.31	**2.48**	2.74	**2.98**	3.47
3-5 years		**1.08**	1.30	**1.52**	1.66	**1.84**	1.99	**2.12**	2.25	**2.39**	2.55	**2.75**	3.05	**3.33**	3.91
5-7 years		**1.19**	1.42	**1.66**	1.81	**2.01**	2.16	**2.30**	2.44	**2.59**	2.76	**2.97**	3.29	**3.59**	4.2
7-9 y.	boys	**1.25**	1.48	**1.73**	1.88	**2.07**	2.22	**2.36**	2.50	**2.65**	2.81	**3.02**	3.33	**3.61**	4.22
	girls	**1.36**	1.61	**1.88**	2.04	**2.25**	2.42	**2.57**	2.72	**2.88**	3.06	**3.28**	3.62	**3.94**	4.58
9-11 y.	boys	**1.47**	1.73	**1.99**	2.15	**2.36**	2.52	**2.66**	2.81	**2.96**	3.14	**3.35**	3.67	**3.97**	4.57
	girls	**1.56**	1.90	**2.20**	2.38	**2.62**	2.80	**2.96**	3.13	**3.30**	3.50	**3.75**	4.11	**4.45**	5.16
11-13 y.	boys	**1.58**	1.88	**2.19**	2.38	**2.63**	2.82	**3.00**	3.18	**3.37**	3.58	**3.84**	4.25	**4.62**	5.39
	girls	**1.62**	1.90	**2.24**	2.46	**2.74**	2.97	**3.17**	3.38	**3.60**	3.85	**4.17**	4.65	**5.10**	6.02
13-15 y.	boys	**1.62**	1.89	**2.24**	2.46	**2.76**	2.99	**3.20**	3.42	**3.65**	3.91	**4.24**	4.75	**5.22**	6.20
	girls	**1.69**	2.03	**2.39**	2.61	**2.91**	3.14	**3.35**	3.56	**3.79**	4.04	**4.36**	4.85	**5.30**	6.24
15-17 y.	boys	**1.70**	2.02	**2.36**	2.57	**2.84**	3.05	**3.25**	3.44	**3.65**	3.88	**4.17**	4.61	**5.01**	5.86
	girls	**1.62**	1.93	**2.26**	2.46	**2.73**	2.93	**3.12**	3.31	**3.51**	3.74	**4.02**	4.45	**4.85**	5.67
17-20 y.		**1.58**	1.90	**2.24**	2.45	**2.72**	2.94	**3.13**	3.33	**3.54**	3.78	**4.07**	4.53	**4.95**	5.83
20-30 y.		**1.55**	1.86	**2.20**	2.41	**2.68**	2.90	**3.09**	3.29	**3.50**	3.74	**4.04**	4.50	**4.92**	5.80
30-40 y.		**1.44**	1.75	**2.08**	2.29	**2.56**	2.78	**2.98**	3.18	**3.39**	3.64	**3.95**	4.42	**4.86**	5.78
40-50 y.		**1.38**	1.68	**2.01**	2.21	**2.48**	2.69	**2.88**	3.08	**3.29**	3.53	**3.83**	4.29	**4.72**	5.63
50-60 y.		**1.34**	1.64	**1.96**	2.16	**2.42**	2.63	**2.83**	3.02	**3.23**	3.46	**3.76**	4.22	**4.65**	5.55
60-70 y.		**1.28**	1.58	**1.90**	2.10	**2.37**	2.58	**2.78**	2.98	**3.19**	3.44	**3.75**	4.23	**4.67**	5.62
70-80 y		**1.20**	1.50	**1.81**	2.00	**2.27**	2.47	**2.67**	2.87	**3.08**	3.32	**3.62**	4.09	**4.52**	5.44
> 80 y		**1.13**	1.43	**1.73**	1.92	**2.19**	2.39	**2.59**	2.79	**3.00**	3.23	**3.54**	4.00	**4.44**	5.36

Serum levels are given as mg/L
Die Serumkonzentrationen sind in mg/L angegeben
week = Woche; months = Monate
y. = years;=Jahre

Determined with IGFBP-3 RIA (Blum et al. 1990)
Mit IGFBP-3-RIA gemessen (Blum et al. 1990)
The values above 70 years are extrapolated.
Die Werte für über 70-Jährige sind extrapoliert.

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SUMMARY – Functional IGFBP-3 LIA E04A

Reconstitution/ Dilution of Reagents:		
Standards A-E	Reconstitution in **Sample Buffer PP** (green)	**1 ml each**
Control Seren KS1 & KS2	Reconstitution in **Sample Buffer PP** (green)	**250 µl**
Ligand Conjugate LK **Enzyme Conjugate EK**	Dilute LK and EK 1:101 each in **Dilution Buffer VP** (e.g. **60 µl LK** plus **6 ml** VP each) (e.g. **120 µl EK** plus **12 ml** VP each)	**1:101 each**
Washing Buffer WP	dilute in **A. dest.** (e.g. add the complete contents of the flask 50 ml into a graduated flask and fill with A.dest. to 1000 ml)	**1:20**
Sample Dilution + Control Sera KS1 & KS2: 1:505 in Sample Buffer PP (green colored), **mix directly and use within max. 60 min.** Use **50 µl per determination** (pipetting control= blue coloration)		
Before assay procedure bring all **reagents** to **room temperature**		

Proposal of Assay Procedure for Double Determination:

Pipette	Reagents	Well Positions
50 µl	diluted Ligand Conjugate **LK**	Pipette **in <u>all</u> required number of wells**
50 µl	Sample Buffer **PP** as Blank	A1 and A2
50 µl	Standard **A** (**0.4 ng/ml**)	B1 and B2
50 µl	Standard **B** (**2 ng/ml**)	C1 and C2
50 µl	Standard **C** (**6 ng/ml**)	D1 and D2
50 µl	Standard **D** (**15 ng/ml**)	E1 and E2
50 µl	Standard **E** (**30 ng/ml**)	F1 and F2
50 µl	diluted Control Serum **KS1**	G1 and G2
50 µl	diluted Control Serum **KS2**	H1 and H2
50 µl	**diluted Samples**	Pipette samples in the rest of the wells according to requirements
Cover the wells with the sealing tape		

Incubation: 2 h at RT, 350 rpm

5x 300 µl	Aspirate the contents of the wells and and wash **5x** with **300 µl** each **WP/ well**	each well
100 µl	diluted Enzyme Conjugate **EK**	each well

Incubation: 1 h at RT, 350 rpm

5x 300 µl	Aspirate the contents of the wells and wash **5x** with **300 µl** each **WP/ well**	each well
100 µl	Substrate Solution **S**	each well

Incubation: 30 min in the dark at RT

100 µl	Stop Solution **SL**	each well
Measure the absorbance within 30 min at **450 nm** (≥590 nm Reference)		

REF E04A  **International Test description**

CAL A-E	**STD A -E**	**Rec in** 1ml **PP**	
Control	**KS1 & KS2**	**Rec in** 250 µl **PP**	**1:505 DILU PP**
LIGAND	**LK**		**1:101 DILU VP**
CONJ	**EK**		**1:101 DILU VP**
WASHBUF 20x	**WP**		**1:20 DILU A. dest.**

SPE	**1:505 DILU PP**
°C 20-25 °C	

50 µl	**LK (1:101 DILU VP)**	A1 - End
50 µl	**PP**	A1/2
50 µl	**CAL A (0.4 ng/ml)**	B1/2
50 µl	**CAL B (2 ng/ml)**	C1/2
50 µl	**CAL C (6 ng/ml)**	D1/2
50 µl	**CAL D (15 ng/ml)**	E1/2
50 µl	**CAL E (30 ng/ml)**	F1/2
50 µl	**CONTROL KS1 1:505 DILU PP**	G1/2
50 µl	**CONTROL KS2 1:505 DILU PP**	H1/2
50 µl	**SPE 1:505 DILU PP**	
TAPE		

2 h °C 20-25 ↔ 350 rpm

5x300 µl	**5x WASHBUF WP**
100 µl	**EK (1:101 DILU VP)**
TAPE	

1 h °C 20-25 ↔ 350 rpm

5x300 µl	**5x WASHBUF WP**
100 µl	**SUBST TMB S**

0.5 h °C 20-25 

100 µl	**H_2SO_4 SL**
MEASURE	