Human IGFBP – 3 total ELISA

(Human Insulin-like Growth Factor Binding Protein-3)

Cat. No.: RMEE03

[IVD] CE

TECHNICAL FEATURES+APPLICATIONS

- Quantitative determination of IGFBP-3 without sample pretreatment

- Inter-Assay variation of 6.30% and Intra-Assay variation of 4.51%

- Sensitivity of 0.1 ng/ml

- Measures growth hormone (GH)-dependent IGFBP-3 Bindingprotein

- 2 Control Sera are provided for quality control purposes according GLP

- Stable serum levels due to absence of circadian variation

- Integrates the GH secretory state over days

- A single measurement is highly informative for diagnosis of GH deficiency or GH excess

- Ideal for the diagnosis of GH-deficiency in young children

- Small sample requirement, thus ideal for pediatric patients.

INTRODUCTION

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, which largely determines the total IGF-I and IGF-II concentration. 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 recognize the complete high molecular weight complex, provided new in-sights into its regulation (6-9). On the basis of these findings serum IGFBP-3 has proved to be an additional useful test in the repertoire of diagnostic tools for evaluation of growth disorders (7,8).

Several factors besides GH influence IGFBP-3 levels: age including sexual development, nutrition, hypothyroidism, diabetes mellitus, liver function and kidney function. 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 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 IGFBP-3 measurements correlate significantly with the logarithm of the integrated spontaneous GH secretion (8,14). In patients with GH deficiency, 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.

So far, IGF-I serum levels have been widely used in screening for GH deficiency or acromegaly.

However, several limitations are obvious:

1. The normal range of IGF-I is low in young children making discrimination of subnormal levels difficult at that age.

2. A considerable number of children of small stature have, despite normal GH secretion, IGF-I levels in the subnormal range. Therefore, the specificity and consequently the accuracy of the test for diagnosis of GH deficiency are limited.

The major advantages of IGFBP-3 over IGF-I are:

1. No extraction step is required prior to measurement (as is still necessary in certain IGF-I assays) thus improving test accuracy by simplifying the assay procedure.

2. The normal range in young children is comparatively high making the detection of subnormal levels more reliable.

3. Patients with GH deficiency have subnormal 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 IGFBP-3 concentration is sufficient for the diagnosis of GH deficiency with high accuracy (7,18). In small statured children IGFBP-3 levels rise to normal range within several days of GH administration and remain normal during continuous GH treatment (Figure 2).

Therefore, 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.

Figure 1: Serum IGFBP-3 levels in patients with short stature without GH deficiency (SS: constitutional delay of growth and adolescence, familial short stature, intra-uterine growth retardation) and in idiopathic or organic GH deficiency (GHD). The normal range is given by the 5th, 50th and 95th percentile.
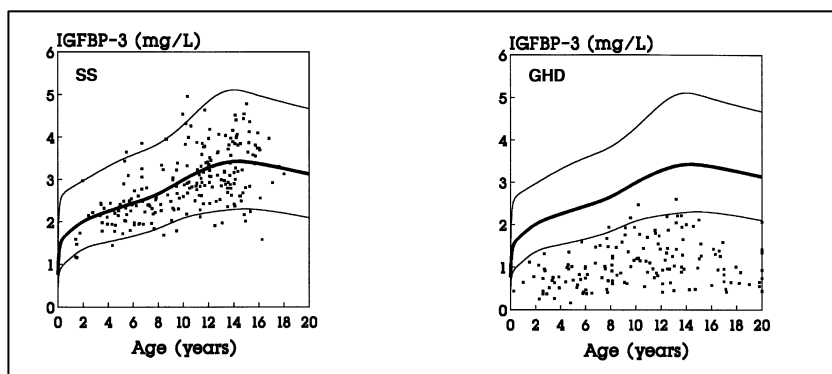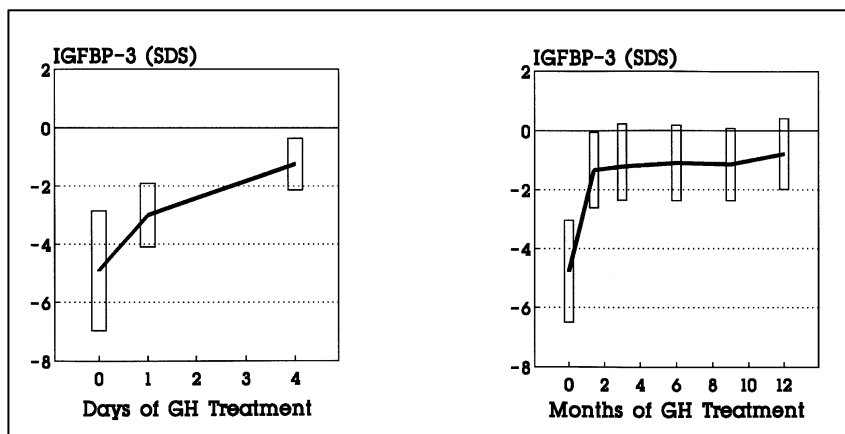

Figure 2: IGFBP-3 levels in GH deficient children before and during GH treatment. Because of the age-dependence, values are given as the mean of standard deviation scores (SDS).

In normal tall children and adolescents without excessive GH secretion or in patients with Sotos syndrome, 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, IGFBP-3 is also a useful parameter for the detection of excessive GH secretion and monitoring therapy efficacy. In precocious puberty, IGFBP-3 levels are clearly increased by chronological age, whereas patients with premature thelarche have IGFBP-3 levels in the upper normal range (15).
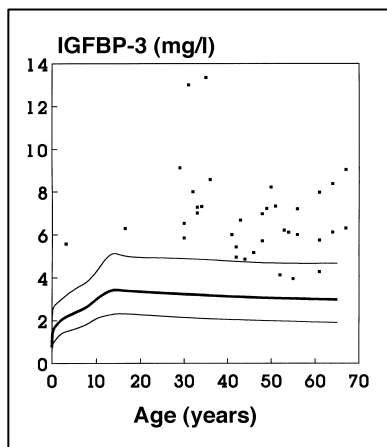
Figure 3: Serum IGFBP-3 levels in acromegaly.

The normal range is given by the 5th, 50th and 95th percentile.

INTENDED USE

This enzyme immunoassay kit is suited for measuring IGFBP-3 in human serum, Heparin-plasma or in cerbrospinal fluid for diagnostic and scientific purposes.

Its diagnostic value for GH deficiency screening is based on the high sensitivity and specificity of serum IGFBP-3 as a test for this diagnosis. States of GH excess may also be detected since IGFBP-3 levels are increased in that case. Due to its constant circadian concentration IGFBP-3 determination in a single blood sample may be sufficient as a screening test for these pathological situations prior to subjecting patients to further testing of GH secretion. IGFBP-3 determinations may also be suited for monitoring the efficacy of treatment and the patient's compliance in GH deficiency and acromegaly.

PERFORMANCE CHARACTERISTICS AND VALIDATION

The ELISA for IGFBP-3 RMEE03 is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The IGFBP3 in the sample binds to the immobilized first antibody on the microtiter plate. In the following step, the biotinylated and Streptavidin-Peroxidase conjugated second specific anti-IGFBP-3-Antibody binds in turn to the immobilised IGFBP-3. In the closing substrate reaction the turn of the colour will be high specific catalysed, quantitatively depending on the IGFBP-3-level of the samples.

The standards of the ELISA RMEE03 are **human IGFBP-3** in concentrations of **0.4; 2; 6; 15 and 30 ng/ml**.

Sensitivity

The **analytical sensitivity** of the ELISA RMEE03 yields **0.1 ng/ml** (2 SD of zero standard in 18 fold determination).

Table 1: Linearity

Dilution:	Sample 1 (recalculated, ng/ml)	Dilution:	Sample 2 (recalculated, ng/ml)
1:20	3250	1:20	3078
1:40	3489	1:40	3179
1:80	3181	1:80	3221
1:160	3167	1:160	3402
1:320	3013	1:320	3066
1:640	2936	1:640	2901
1:1280	2895	1:1280	3364
AV / 1SD / VC%	3133 / 205 / 6,54	**AV / 1SD / VC%**	3173 / 176 / 5.55

AV = Average Value , SD = Standard Deviation; VC = Coefficient of VariationTable

The **Inter**- and **Intra-Assay** variation coefficients were found less than **6.30 % and 4.51 %**. Exemplary determinations are shown in table 2 and table 3.

Table 2: Inter-Assay-Variation

	Mean Value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	2568	148	5.76
Sample 2	3334	210	6.30
Sample 3	4082	233	5.70

Table 3: Intra-Assay-Variation

	Mean Value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	1764	76.6	4.34
Sample 2	2260	98.5	3.96
Sample 3	3699	167.0	4.51

Clinical validation was achieved by determination of IGFBP-3 levels in a large number of normal children and adults, normal short statured children without GH deficiency, girls with Ullrich-Turner Syndrome, children with Silver-Russell Syndrome, patients with GH deficiency, children with familial tall stature, Sotos-Syndrome, patients with acromegaly, children with premature thelarche and precocious puberty (Tab. 4; Abb. 1, 2, 3, 4 und 5).

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

Serum samples, Heparin-Plasma samples and Cerebrospinalfluid samples are suitable. A special external sample preparation prior to assay is not required. Results in Citrat- or EDTA-Plasma are about 15% reduced. Slight Hemolysis of the samples doesn´t disturb the determination.

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 IGFBP-3 levels were found to be unaffected by few cycles (5x) in our experiments.

The high sensitivity of the assays allows IGFBP-3 determinations in small sample volumes, which is limited by pipetting accuracy rather than the amount of IGFBP-3.

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**, the respective covered range would be 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** (red 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 = red coloring of the solution in the wells).

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 IGFBP-3 Antibody, packed in a laminate bag.
2.	CAL	**Standards A-E**, lyophilised, contain human IGFBP-3. Standard values are between 0.4 - 30 ng/ml (0.4, 2, 6, 15 and 30 ng/ml) IGFBP-3, Standards are **reconstituted with 1 ml Sample Buffer PP each**. Use 50 µl pro well in the assay.
3.	BUF VP	**Dilution Buffer VP**, 30 ml, ready for use. **Please shake before use!** Use 50 µl pro well in the assay.
4.	BUF PP	**Sample Buffer PP**, 120 ml, ready for use, red colored, please use for the reconstitution of **Standards (A-E)** and **Controls** and for dilution of **Samples** and **Controls**
5.	Control	**Control Sera KS1 and KS2**, 250 µl, lyophilised, contain human Serum and should be **reconstituted in each 250 µl Sample Buffer PP**. The IGFBP-3 target values and the respective ranges are given on the vial labels. The dilution should be according to the dilution of the respected samples. Use 50 µl pro well in the assay.
6.	Ab CONJ	**Antibody-HRP-Conjugate AK**, **12 ml, ready for use**, contains a mixture of biotinylated anti-human IGFBP-3 Antibody and HRP (Horseradish Peroxidase)-labelled Streptavidin. Use 100 µl pro well in the assay.
7.	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.
8.	SUBST	**Substrate (S),** 12 ml, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbencidine.
9.	H_2SO_4	**Stopping Solution (SL),** 12 ml, ready for use, 0.2 M sulphuric acid, Caution acid!
10.		Sealing tape for covering of the microtiter plate, 2 x, adhesive.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes 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)

- Reservoirs (disposable)

- Plate washer and plate shaker

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

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) 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 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, 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.

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 pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, the **Antibody-POD-Conjugate AK**, **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 pipette on before in **all needed wells 50 µl Dilution Buffer VP**.

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

3) Pipette in positions B1/2 **50 µl** each **Standard A (0.4 ng/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 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.**

4) Cover the wells with the sealing tape and incubate the plate for **1 hour** at **room temperature** ¨ (shake at ≥350 rpm).

5) After incubation aspirate the contents of the wells and wash the wells 5 times with **250 µl Washing Buffer WP**.

6) Following the last washing step pipette **100 µl** of the **Antibody-POD-Conjugate AK** in each well.

7) Cover the wells with the sealing tape and incubate **1 hour** at **room temperature** (shake at ≥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 TMB-Substrate solution S** in each well.

10) Incubate the plate for **30 Minutes in the dark at** room **temperature**.

11) After incubation pipette **100 µl Stop Solution SL** in each well.

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 concentrations of hIGFBP-3

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

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 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).

EXPECTED VALUES

IGFBP-3-levels are strongly age-dependent in children, less so in adults. The normal ranges in various age-groups which were log-normally distributed are given in table 4 by the percentiles (see Appendix). A graphic presentation is shown in Fig.4 and 5. It is recommended for each laboratory to establish its own normal range.

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SUMMARY –IGF-II ELISA RMEE30

Reconstitution / Dilution of Reagents		
Standards A-E	Reconstitution in **Sample Buffer PP** (red)	**1 ml each**
Control Serum KS1 & KS2	Reconstitution in **Sample Buffer PP** (red)	**250 µl 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 (red colored), **mix directly and use within max. 60 min.** Use **50 µl per determination** (pipetting control= red coloration)		
Before assay procedure bring all **reagents** to **room temperature**		

Proposal of Assay Procedure for Double Determination:

Pipette	Reagents	Well Positions
50 µl	Dilution Buffer **VP**	Pipette **in all 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	Control Serum **KS1**	G1 and G2
50 µl	Control Serum **KS2**	H1 and G2
50 µl	**Sample**	Pipette sample in the rest of the wells according to requirements
Cover the wells with the sealing tape		

Incubation: 1 h at RT (shake at ≥350 rpm)

5x 250 µl	Aspirate the contents of the wells and wash **5x** with **250 µl** each **WP**/**well**	each well
100 µl	Antibody-POD-Conjugate **AK**	each well

Incubation: 1 h at RT (shake at ≥350 rpm)

5x 250 µl	Aspirate the contents of the wells and wash **5x** with **250 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)		

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 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.

ESTABLISHING THE STANDARD CURVE

The standards provided contain the following concentrations of hIGFBP-3

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

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 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 OF PROCEDURE

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 seen in Western ligand blotting, but in general only have little influence on the outcome of ELISA determinations. In case of special interest in this physiological process, the ELISA for **functional IGFBP-3 E04** is available. The ELISA E04 enables to quantify the degree of **IGFBP-3 fragmentation** in samples.

APPENDIX

Table 4: Serum levels of 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
y. = years

Determined with IGFBP-3 RIA (Blum et al. 1990)
The values above 70 years are extrapolated.
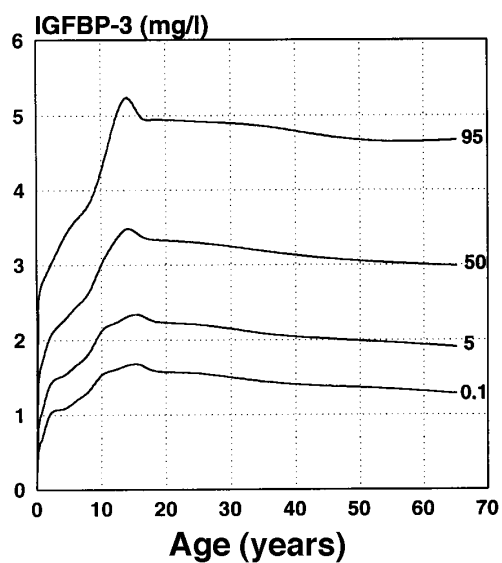
Serum conc. according to age

IGFBP-3 (mg/l)

Age (years)

Fig. 5: Age-dependant normal values of IGFBP-3
(presented as 0.1., 5., 50., and 95. percentile)

Children and adolescents

IGFBP-3 (mg/l)
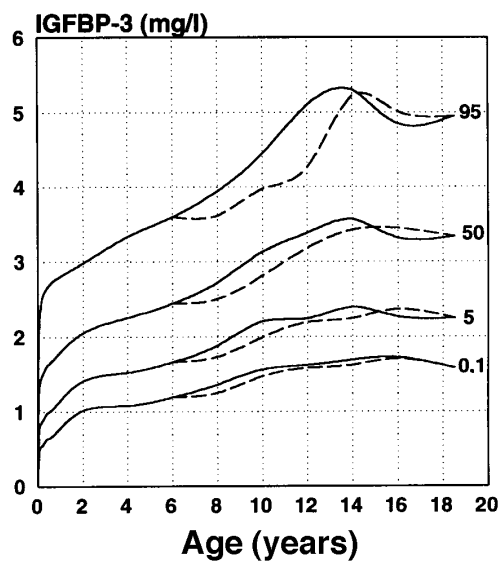

Age (years)

Fig. 6: Normal values of children and adolescents
(girls ——— boys - - - -)

REF RMEE03   [**i**]   **International Test Description**

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

Control						1:505	**DILU**	PP
SPE						1:505	**DILU**	PP
°C	**20-25 °C**							

50 µl	**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	

🕐 **1 h**   **°C 20-25**   **≥ 350 rpm**   [↔]

5x 250 µl	**5x WASHBUF WP**
100 µl	**AbCONJ AK**
	TAPE

🕐 **1h**   **°C 20-25**   **≥ 350 rpm**   [↔]

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

🕐 **0.5 h**   **°C 20-25**   ☀

100 µl	**H₂SO₄** **SL**
	MEASURE

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