Human IGFBP – 3 total ELISA
(Human Insulin-like Growth Factor Binding Protein-3)

Cat. No.: RMEE03A

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 Binding protein
- 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)-1 and -2 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, ... IGBPBP-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-1 and IGF-2 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-1 or IGF-2 (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 insights 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-1, 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-1 serum levels have been widely used in screening for GH deficiency or acromegaly. However, several limitations are obvious:

1. The normal range of IGF-1 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-1 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-1 are:

1. No extraction step is required prior to measurement (as is still necessary in certain IGF-1 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 cerebrospinal 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 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 RMEE03A are human IGFBP-3 in concentrations of 0.4; 2; 6; 15 and 30 ng/ml.
Sensitivity

The analytical sensitivity of the ELISA RMEE03A yields 0.1 ng/ml (2 SD of zero standard in 18-fold determination).

Table 1: Linearity

<table>
<thead>
<tr>
<th>Dilution:</th>
<th>Sample 1 (recalculated, ng/ml)</th>
<th>Sample 2 (recalculated, ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:20</td>
<td>3250</td>
<td>3078</td>
</tr>
<tr>
<td>1:40</td>
<td>3489</td>
<td>3179</td>
</tr>
<tr>
<td>1:80</td>
<td>3181</td>
<td>3221</td>
</tr>
<tr>
<td>1:160</td>
<td>3167</td>
<td>3402</td>
</tr>
<tr>
<td>1:320</td>
<td>3013</td>
<td>3066</td>
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<tr>
<td>1:640</td>
<td>2936</td>
<td>2901</td>
</tr>
<tr>
<td>1:1280</td>
<td>2895</td>
<td>3364</td>
</tr>
</tbody>
</table>

AV / 1SD / VC% = 3133 / 205 / 6,54

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

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Value (ng/ml)</th>
<th>Standard Deviation (ng/ml)</th>
<th>VC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>2568</td>
<td>148</td>
<td>5.76</td>
</tr>
<tr>
<td>Sample 2</td>
<td>3334</td>
<td>210</td>
<td>6.30</td>
</tr>
<tr>
<td>Sample 3</td>
<td>4082</td>
<td>233</td>
<td>5.70</td>
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</tbody>
</table>

Table 3: Intra-Assay-Variation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Value (ng/ml)</th>
<th>Standard Deviation (ng/ml)</th>
<th>VC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>1764</td>
<td>76.6</td>
<td>4.34</td>
</tr>
<tr>
<td>Sample 2</td>
<td>2260</td>
<td>98.5</td>
<td>3.96</td>
</tr>
<tr>
<td>Sample 3</td>
<td>3699</td>
<td>167.0</td>
<td>4.51</td>
</tr>
</tbody>
</table>

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 (yellow 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).
## REAGENTS PROVIDED

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>1.</td>
<td>MTP</td>
</tr>
<tr>
<td>2.</td>
<td>CAL</td>
</tr>
<tr>
<td>3.</td>
<td>BUF VP</td>
</tr>
<tr>
<td>4.</td>
<td>BUF PP</td>
</tr>
<tr>
<td>5.</td>
<td>Control</td>
</tr>
<tr>
<td>6.</td>
<td>Ab CONJ</td>
</tr>
<tr>
<td>7.</td>
<td>WASHBUF 20X</td>
</tr>
<tr>
<td>8.</td>
<td>SUBST</td>
</tr>
<tr>
<td>9.</td>
<td>H₂SO₄</td>
</tr>
<tr>
<td>10.</td>
<td></td>
</tr>
</tbody>
</table>

## 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
- Polyethylene PE/Polypropylene 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 microtiter 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 microtiter plate washer, the respective instructions for 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 dynamically swinging out the microtiter 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 Controls
For the reconstitution of the lyophilised components (Standards A - E and Control Sera KS1 & KS2) the kit Sample Buffer PP has to be used. It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.

The reconstituted standards and controls can be stored for 3 months at –20°C. Repeated freeze/thaw cycles have to be avoided. When using the standards anew, please thaw them rapidly but gently (no temperature rise over the room temperature and no powerful vortexing), 3 of these freezing-thawing cycles showed no influence on the assay.

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

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

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.

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
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$_2$SO$_4$)**

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 300 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).

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.

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.
REFERENCES


SUMMARY –IGF-2 ELISA RMEE30

| Standards A-E | Reconstitution in Sample Buffer PP (yellow) | 1 ml each |
| Control Serum KS1 & KS2 | Reconstitution in Sample Buffer PP (yellow) | 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 (yellow 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:

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Reagents</th>
<th>Well Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µl</td>
<td>Dilution Buffer VP</td>
<td>Pipette in all required number of wells</td>
</tr>
<tr>
<td>50 µl</td>
<td>Sample Buffer PP as Blank</td>
<td>A1 and A2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Standard A (0.4 ng/ml)</td>
<td>B1 and B2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Standard B (2 ng/ml)</td>
<td>C1 and C2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Standard C (6 ng/ml)</td>
<td>D1 and D2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Standard D (15 ng/ml)</td>
<td>E1 and E2</td>
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<tr>
<td>50 µl</td>
<td>Standard E (30 ng/ml)</td>
<td>F1 and F2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Control Serum KS1</td>
<td>G1 and G2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Control Serum KS2</td>
<td>H1 and G2</td>
</tr>
<tr>
<td>50 µl</td>
<td>Sample</td>
<td>Pipette sample in the rest of the wells according to requirements</td>
</tr>
</tbody>
</table>

Cover the wells with the sealing tape

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

| 5x 300 µl | Aspirate the contents of the wells and wash 5x with 300 µl each WP/well | each well |
| 100 µl    | Antibody-POD-Conjugate AK | each well |

**Incubation: 1 h at RT (shake at 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)

**ESTABLISHING THE STANDARD CURVE**

The standards provided contain the following concentrations of hIGFBP-3

<table>
<thead>
<tr>
<th>Standard</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/ml</td>
<td>0.4</td>
<td>2</td>
<td>6</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>

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 RME04 is available. The ELISA RME04 enables to quantify the degree of IGFBP-3 fragmentation in samples.


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.

<table>
<thead>
<tr>
<th>Altersgruppe</th>
<th>0.1</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
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<tbody>
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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.
Serum conc. according to age

![Graph showing serum concentration of IGFBP-3 (mg/l) according to age.]

Children and adolescents

![Graph showing serum concentration of IGFBP-3 (mg/l) according to age for children and adolescents.]

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

Fig. 6: Normal values of children and adolescents (girls — boys ——).
<table>
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<th>A-E</th>
<th>A-E</th>
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<th>PP</th>
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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 1 h °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