Human IGFBP-3 ELISA

(functional Insulin-Like Growth-Factor Binding Protein-3)

Cat. No.: RMEE04R

CLINICAL RELEVANCE

This Ligand-binding Immunoassay kit is suited for measuring human bioactive and functional - IGF-I binding - IGFBP-3.

In conjunction with correspondent measurements of total IGFBP-3 with respective kits RMEE03, IGF-R10, or, IGF-R11, the degree of fragmentated IGFBP-3 can be determined.

TECHNICAL PROPERTIES AND APPLICATIONS

♦ 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 (E03, IGF-R10, IGF-R11). In retrospect, or, in simultaneous determinations out of the same sample dilution!
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 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 (t-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, ... IGFBP-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 (f-IGFBP-3)**

By proteolytical cleavage of the ternary complex of IGFBP-3, the physiological storage of IGF-I in circulation, IGF-I is 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.

An increase in IGFBP-3 fragmentation is seen in:
- Growth Hormone Receptor Insensitivity (22, 23),
- Catabolic States like sepsis, traumatic and postoperative states (24-26),
- Non-Insulin-Dependent Diabetes (27),
- Burns (28),
- Cancer, i.e., breast cancer (29), colorectal cancer(30), tumor of the nervous system of children (31).

A decrease in IGFBP-3 fragmentation is seen in the synovial fluid of arthritic patients (32).

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, this part was quantitated more elevated in samples of acromegalic, IDDM and NIDDM patients (33, 34).

**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 the 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-Peroxidase-(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 quantitated!
The standards of the LIA RMEE04R are made of native and functional human IGFBP-3 in concentrations of 2, 10, 30, 75 and 150 ng/ml.

The analytical sensitivity of the LIA RMEE04R has been determined with 0.18 ng/ml (2 SD of zero standard in 16 fold determination).

The IGFBP-3 LIA RMEE04R 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**

( representative results of two different sera are listed)

<table>
<thead>
<tr>
<th>Dilution:</th>
<th>Sample 1 (recalculated, ng/ml)</th>
<th>Dilution:</th>
<th>Sample 2 (recalculated, ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:50</td>
<td>1675</td>
<td>1:50</td>
<td>2419</td>
</tr>
<tr>
<td>1:100</td>
<td>1768</td>
<td>1:100</td>
<td>2299</td>
</tr>
<tr>
<td>1:150</td>
<td>1735</td>
<td>1:150</td>
<td>2314</td>
</tr>
<tr>
<td>1:200</td>
<td>1788</td>
<td>1:200</td>
<td>2310</td>
</tr>
<tr>
<td>1:250</td>
<td>1834</td>
<td>1:250</td>
<td>2396</td>
</tr>
<tr>
<td>1:300</td>
<td>1902</td>
<td>1:300</td>
<td>2422</td>
</tr>
</tbody>
</table>

AV / SD / VC% = 1784 / 79 / 4.4  
AV / SD / VC% = 2360 / 58 / 2.5

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)**

<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>1051</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1891</td>
<td>129</td>
<td>6.8</td>
</tr>
<tr>
<td>Sample 3</td>
<td>2417</td>
<td>158</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Table 3: Intra-Assay-Variation (n=16)

<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>1061</td>
<td>26</td>
<td>2.5</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1571</td>
<td>85</td>
<td>5.4</td>
</tr>
<tr>
<td>Sample 3</td>
<td>2660</td>
<td>148</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Clinical Validation

TOTAL IGFBP-3

Clinical validation was achieved by determination of total immunoreactive IGFBP-3 levels in a large number of normal children and adults, normal short stunted 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. 5; Abb. 1, 2, 3, 6 und 7). Based on these studies the respective kits for measurement of total human immunoreactive IGFBP-3 (i.e., RMEE03, IGF-R10, IGF-R11) are CE-approved for the clinical diagnostic use.

FRAGMENTED IGFBP-3

The results determined with the human IGFBP-3 LIA RMEE04R 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 RMEE04R for functional IGFBP-3 and for total immunoreactive IGFBP-3 with 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 females were found lower than those of males. 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 24 % lower (Tab. 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.

<table>
<thead>
<tr>
<th></th>
<th>Total IGFBP-3; min/max (ng/ml)</th>
<th>Funct. IGFBP-3; min/max (ng/ml)</th>
<th>Functional IGFBP-3 (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>3568; 1752/5752</td>
<td>2506; 1102/4346</td>
<td>70,2</td>
</tr>
<tr>
<td>Males</td>
<td>2752; 1258/4726</td>
<td>2332; 1337/4304</td>
<td>84,8</td>
</tr>
<tr>
<td>Overall</td>
<td>3160</td>
<td>2419</td>
<td>77,5</td>
</tr>
</tbody>
</table>

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, RMEE04R 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 Mediosk GmbH is not liable for any loss or harm caused by non-observance of the instructions, as far as no law withstands.

Before use, all kit components should be brought to room temperature at 20 - 25°C. Precipitates in buffers should be dissolved before use by thorough mixing and warming. **Temperature WILL affect the absorbance** readings of the assay. However, Values for the patient samples will not be affected.

Do not mix reagents of different lots. Do not use expired reagents.

The microplate contains snap-off strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.

Caution: This kit contains material of human and/or animal origin. Source human serum for the Control Serum provided in this kit 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) antibody. 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 (H₂SO₄)**

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.

Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step. 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. Some reagents contain <0.1% Kathon CG, <0.1% ProClin 950, as preservatives. In case of contact with eyes or skin, flush immediately with 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 eyes, rinse 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 IGFBPr3.

2. **CAL**
   - **Standards A-E**, 250 µl, lyophilised, contain native human functional IGFBPr3. Standard values are between 2 - 150 ng/ml (2, 10, 30, 75 and 150 ng/ml) functional IGFBPr3. Standards are to be reconstituted with 250 µl Sample Buffer PP each. Use 10 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 the dilution of the Samples, Standards and Control.

5. **Control**
   - **Control Serum KS**, 250 µl, lyophilised, contains human Serum and should be reconstituted in 250 µl Sample Buffer PP. The functional IGFBP-3 target value and the respective range are given on the vial label. The dilution should be according to the dilution of the respected samples. Use 10 µl per well in the assay.

6. **Ligand**
   - **Ligand Conjugate LK**, 140 µl, 101-fold Concentrate, contains biotinylated recombinant human IGFr-I. Before use dilute 1:101 with VP. Use 100 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 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 limited stable, please dilute only according to requirements.

9. **SUBST**
   - **Substrate (S)**, 12 ml, ready for use, horseradish- peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbenzidine.

10. **H₂SO₄**
    - **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 (100 and 200µl) 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)
- Timer (120 min. range)
- Reservoirs (disposable)
- Plate washer and plate shaker (recommended)
- Calibrated Micro plate reader ("ELISA-Reader") with filter for 450 and 620nm (or ≥ 590 nm)
- Foil welding device for laminate bags (recommended)
REAGENT PREPARATION

Bring all reagents to room temperature (20 - 25°C) before use.

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 limited stable, dilute only according to requirements. This applies to the 1:101 dilutions of Ligand Conjugate LK and Enzyme Conjugate EK too.

The Standards A – E and Control Serum KS 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.

Use the Dilution Buffer VP for the dilution of Ligand and Enzyme Conjugate concentrates (LK and EK).

STORAGE CONDITIONS

The microtiter plate wells and all undiluted reagents are stable until the expiry date, if stored in the dark at 2-8°C.

Store the unused seal strips and microtiter wells together with the desiccant at 2° to 8°C.

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

Reconstituted components (Standards (A – E) and Control Serum (KS)) 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.

Figure 3: Serum IGFBP-3 levels in acromegaly. The normal range is given by the 5th, 50th and 95th percentile.

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

Serum samples, Heparin- and EDTA-Plasma samples are suitable. Citrat-Plasma samples are not recommended, values are reduced. A special external sample preparation prior to assay is not required. Citrat-Plasma samples are not recommended, values are reduced.

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 1:101 with Sample Buffer PP is suitable, thus the respective covered assay range is 0.2 to 15.15 mg/L (or, 200 to 15,150 ng/ml). Where required, depending on the expected IGFBP-3-values, the dilution with Sample Buffer PP can be higher or lower, we recommend to use dilutions in the range from 1:50 to 1:300. The IGFBP-3 concentrations might 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) and mix each tube immediately. After mixing, use 10 µl of this solution per determination within 1 hour 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.

1) Please dilute the Ligand Conjugate LK 1:101 with Dilution Buffer VP (for use of the entire Microtitre Plate you may need 9.6 ml, thus you may dilute 120 µl of concentrated LK with 12 ml of buffer VP).
2) Add in every needed well 100 µl of the already 1:101 diluted Ligand Conjugate LK
3) Add 10µl Sample Buffer PP in positions A1/2 (=blank wells !)
4) Pipette in positions B1/2 10µl each Standard A (2 ng/ml),
   pipette in positions C1/2 10µl each Standard B (10 ng/ml),
   pipette in positions D1/2 10µl each Standard C (30 ng/ml),
   pipette in positions E1/2 10µl each Standard D (75 ng/ml),
   pipette in positions F1/2 10µl each Standard E (150 ng/ml),
To control the correct test accomplishment 10 µl of the 1:101 (or in respective dilution rate of the sample) in Sample Buffer PP diluted Control Serum KS can be pipetted in positions G1/2.
   Pipette 10 µl each of the diluted sample (generally 1:101 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 (if possible, shake at ≥350 rpm).
6) After incubation aspirate the contents of the wells and wash the wells 3 times with 250 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 (if possible shake at ≥350 rpm).

9) After incubation wash the wells 3 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, e.g. 620 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 exceed 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 f-IGFBP-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>2</td>
<td>10</td>
<td>30</td>
<td>75</td>
<td>150</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. **Non-linear regression**, a higher-grade polynomial or four parametric logistic (4-PL) lin-log curve fit are suitable for the evaluation. A good fit is provided with cubic spline, 4 Parameter Logisitcs or Logit-Log.
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 30 ng/ml, Sample was 1:101 diluted: 30 x 101= 3030 ng/ml, or 3,03 µg/ml or 3,03 mg/L, according the requested unit).

**EXPECTED VALUES** TOTAL IGFBP-3
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 5 by the percentiles. A graphic presentation is shown in **Fig.6 and 7**. It is recommended for each laboratory to establish its own normal range.

**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.
EXPECTED VALUES FUNCTIONAL IGFBP-3

It is recommended for each laboratory to establish its own normal range for functional IGFBP-3. The given t-IGFBP-3 normal values (Tab.5) might be used for an estimation of the expected values as well as for quantifying the degree of IGFBP-3 fragmentation in samples. In case of comparative studies of the ratio total to functional IGFBP-3 concentrations they are the basis for the total immunoreactive part of IGFBP-3.
Appendix

Figure 1: Total 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: Total 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).
Figure. 4: Plot of functional IGFBP-3 E04 values against total IGFBP-3 values of sera from 212 healthy blood donors (IGFBP-3 concentrations in ng/ml).

Fig. 5: Plot of functional IGFBP-3 E04 values against total IGFBP-3 values of sera from 72 sickened donors (IGFBP-3 concentrations in ng/ml; sera of several growth disorders, cancers and liver diseases).
REFERENCES


32) Fernihough JK, B.M., Cwyfan-Hughes S, Holly JMP. Local disruption of the insulin-like growth factor system in the arthritic joint. Arthritis Rheum 39, 1556-1565 (Local disruption of the insulin-like growth factor system in the arthritic joint.).
### SUMMARY – functional IGFBP-3 LIA RMEE04R

#### Reconstitution/ Dilution of Reagents:

<table>
<thead>
<tr>
<th>Standards A-E</th>
<th>Reconstitution in Sample Buffer PP (green)</th>
<th>250 µl each</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Serum KS</td>
<td>Reconstitution in Sample Buffer PP (green)</td>
<td>250 µl</td>
</tr>
<tr>
<td>Ligand Conjugate LK</td>
<td>Dilute LK and EK 1:101 each in Dilution Buffer VP (e.g. 120 µl LK or EK, plus 12 ml VP each)</td>
<td>1:101 each</td>
</tr>
<tr>
<td>Enzyme Conjugate EK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washing Buffer WP</td>
<td>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)</td>
<td>1:20</td>
</tr>
</tbody>
</table>

**Sample Dilution + Control Serum KS**: 1:101 in Sample Buffer PP (green colored; e.g. 10 µl in 1 ml PP), mix directly and use within max. 60 min.

Use 10 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 Reagents</th>
<th>Well Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µl</td>
<td>diluted Ligand Conjugate LK</td>
</tr>
<tr>
<td>10 µl</td>
<td>Sample Buffer PP as Blank</td>
</tr>
<tr>
<td>10 µl</td>
<td>Standard A (2 ng/ml)</td>
</tr>
<tr>
<td>10 µl</td>
<td>Standard B (10 ng/ml)</td>
</tr>
<tr>
<td>10 µl</td>
<td>Standard C (30 ng/ml)</td>
</tr>
<tr>
<td>10 µl</td>
<td>Standard D (75 ng/ml)</td>
</tr>
<tr>
<td>10 µl</td>
<td>Standard E (150 ng/ml)</td>
</tr>
<tr>
<td>10 µl</td>
<td>diluted Control Serum KS</td>
</tr>
<tr>
<td>10 µl</td>
<td>diluted Samples</td>
</tr>
</tbody>
</table>

Cover the wells with the sealing tape

**Incubation**: 2 h at RT, ≥ 350 rpm

| 3x 250 µl | Aspirate the contents of the wells and wash 3x with 250 µl each WP/well | each well |
| 100 µl | diluted Enzyme Conjugate EK | each well |

**Incubation**: 1 h at RT, ≥350 rpm

| 3x 250 µl | Aspirate the contents of the wells and wash 3x 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)