Version 18-11/24

User's Manual

Human IGFBP-3 (insulin-like growth factor binding protein 3) ELISA Kit

REF **DEIA-NB24-14**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

This enzyme immunoassay kit is suited for measuring IGFBP-3 in human serum, EDTA and Heparin-plasma

General Description

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. 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 property to associate with an acid-labile subunit (ALS) after binding of either IGF-I or IGF-II. Most of the IGFBP-3 in plasma is present as high molecular weight ternary complex, however, small amounts of free IGFBP-3 are also found.

The development of a specific immunoassays for IGFBP-3, which detects IGFBP-3 in the ternary complex, provided new in-sights into IGFP-3 regulation. On the basis of these findings serum IGFBP-3 has been proven to be an additional useful test in the repertoire of diagnostic tools for evaluation of growth disorders.

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, but are increased in chronic renal failure. Measurement over 24 hours revealed no circadian rhythm. 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. In patients with GH deficiency, IGFBP-3 levels are subnormal and increase gradually to within the normal range after several days of GH administration. The slow response to GH and constant circadian levels during chronic daily application of GH suggest that IGFBP-3 reflects the GH secretory state over days.

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 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.

Principles of Testing

The Human IGFBP-3 ELISA Kit is a so-called Sandwich-Assay. It utilizes two specific antibodies of high affinity. First the IGFBP-3 in the sample binds to the immobilized antibody on the microtiter plate. In the following step, the complex of biotinylated anti-IGFBP3-Antibody and Streptavidin-Peroxidase binds in turn to the immobilised IGFBP-3. Subsequently, the peroxidase catalyzes an enzymatic reaction resulting in a blue coloration. The intensity of the blue color depends on the IGFBP-3 content of the sample. The reaction is stopped by the addition of stop solution and color intensity is quantified by measuring the absorption.

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with rabbit-anti-hIGFBP-3-antibody. Wells are separately breakable.

(8x12) wells

2. **Calibrators (CAL A-E):** lyophilized, (human IGFBP-3), concentrations are given on vial labels and on quality certificate. 5 x 1 mL
3. **Control 1 (CTR1):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 250 µL
4. **Control 2 (CTR2):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 250 µL
5. **Antibody-HRP-Conjugate (DET):** ready for use, contains rabbit biotinylated anti-hIGFBP-3 antibody. 1 x 12 mL
6. **Sample Buffer (SB):** red color, ready for use. Please shake before use! 1 x 120 mL
7. **Dilution Buffer (DIL):** ready for use. Please shake before use! 1 x 30 mL
8. **Washing Buffer (WB):** 20-fold concentrated solution. 1 x 50 mL
9. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised H_2O_2 Tetramethylbencidine. 1 x 12 mL
10. **Stopping Solution (STP):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
11. **Sealing Tape:** for covering the microtiter plate. 2

Materials Required But Not Supplied

1. Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer WP (A. dest.), 950 mL.
2. Precision pipettes and multichannel pipettes with disposable plastic tips
3. Polyethylene PE/Polypropylene PP tubes for dilution of samples
4. Vortex-mixer
5. Microtiter plate shaker (350 rpm)
6. Microtiter plate washer (recommended)
7. Microplate reader ("ELISA-Reader") with filter for 450 and ≥590 nm

Storage

Store the kit at 2-8°C after receipt until its expiry date. The lyophilized reagents should be stored at –20 °C after reconstitution. Avoid repeated thawing and freezing.

The shelf life of the components after initial opening is warranted for 4 weeks, store the unused strips and microtiter wells airtight together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The reconstituted components Calibrators A-E and Controls CTR1 and CTR2 must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Up to 3 of the freeze-thaw cycles did not influence the assay. The 1:20 diluted Washing Buffer WP is 4 weeks stable at 2-8°C.

Specimen Collection And Preparation

1. Sample type: Serum and Plasma

Serum and Heparin/EDTA Plasma yield comparable values.

2. Specimen collection

Use standard venipuncture for the blood sampling. Haemolytic reactions have to be avoided.

3. Required sample volume: 10 µL

4. Sample stability

In firmly closable sample vials

• Storage at 20-25°C: 3 days

• Storage at -20° C: min. 2 years

• Freeze-thaw cycles max. 10

The storage of samples over a period of 2 years at -20°C, showed no influence on the reading. Freezing and thawing of samples should be minimized.

5. Interference

Triglyceride, bilirubin and hemoglobin in the sample do not interfere to a concentration of 100 mg/mL, 100 µg/mL or 5 mg/mL, respectively. However, the use of haemolytic, lipemic or icteric samples should be validated by the user.

6. Sample dilution

• Dilution: 1:505 with Sample Buffer SB

• Pipette 1 ml Sample Buffer SB (red colored) in PE-/PP-Tubes (application of a multistepper is recommended in larger series), add 10 µL Serum- or Plasma (dilution factor 101). Add 400 µL Sample Buffer SB in another PE-/PP-tube and 100 µL of the thoroughly mixed first dilution (dilution factor 5). After mixing use 50 µL of this 1:505 diluted solution within 1 hour per determination in the assay.

• Sample stability after dilution of the sample: maximum 1 hour at 20-25°C.

Reagent Preparation

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

1. The Calibrators A – E is reconstituted with 1 mL Sample Buffer SB. After resuspension, the calibrators are diluted according to a gradient - A (0.4 ng/mL), B (2 ng/mL), C (6 ng/mL), D (15 ng/mL), E (30 ng/mL), which are prepared for immediate use.
2. The Controls CTR1 and CTR2 are reconstituted with 250 µL Sample Buffer SB. After reconstitution dilute the Controls CTR1 and CTR2 with the Sample Buffer SB in the same ratio (1:505) as the sample.

Note: 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.

3. The required volume of Washing Buffer WB is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest.

Assay Procedure

Note

1. Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate S, stabilised Tetramethylbencidine, is photosensitive–store and incubation in the dark.

2. When performing the assay, Blank, Calibrators A-E, Controls CTR1 and CTR2 and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody-HRP-Conjugate DET as well as the succeeding Substrate S should be added to the plate in the same order and in the same time interval as the samples. Stop Solution STP should be added to the plate in the same order as Substrate S. All determinations (Blank, Calibrators A-E, Controls CTR1 and CTR2 and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
3. Shaking: The incubation steps should be performed at mean rotation frequency of a microtiter plate shaker. We recommend 350 rpm. Depending on the design of the shaker, the shaking frequency should be adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/ or false values, excessive shaking may result in high optical densities and/ or false values.
4. 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 WB diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least.

   Automatic washing: When using an Automatic microtiter plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

   Manual washing: Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical 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.

Assay Step

1. Set Calibrator A-E, test samples (1:505 diluted), Controls CTR1 and CTR2 (1:505 diluted) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each calibrator and sample in duplicate.
2. Add 50ul Dilution Buffer DIL in all wells used. Aliquot 50ul of Sample Buffer SB (Blank), Calibrator A-E, Controls CTR1 and CTR2 and test samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 20-25°C, 350 rpm for 1h.
4. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WB. Do not let the wells dry completely at any time.
5. Add 100ul Antibody-HRP-Conjugate DET into above wells. Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 20-25°C, 350 rpm for 1h.
6. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WB. Do not let the wells dry completely at any time.
7. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark for 30 minutes.

8. Stop: Add 100ul Stopping Solution STP into each well.
9. Measure the absorbance within 30 min at 450 nm, with ≥ 590 nm as reference wavelength.

Quality Control

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

Quality criteria

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.25, and the absorbance of Calibrator E should be above 1.00. Samples, which yield higher absorbance values than Calibrator E, should be re-tested with a higher dilution.

Calculation

1. Calculate the mean absorbance value for the blank from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance of the blank from the mean absorbances of all other samples and calibrators.
3. Plot the calibrator concentrations on the x-axis versus the mean value of the absorbance of the calibrators on the y-axis.
4. Recommendation: Calculation of the calibration 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 (or pg/mL, according the chosen unit for the calibrators) of the samples can be calculated by multiplication with the respective dilution factor.

Exemplary calculation of IGFBP-3 concentrations

Sample dilution: 1:505

Measured extinction of your sample 0.975

Measured extinction of the blank 0.204

Your measurement program will calculate the IGFBP-3 concentration of the diluted sample automatically by using the difference of sample and blank for the calculation. You only have to determine the most suitable curve fit (here: polynomial 3rd degree).

In this exemplary case the following equation is solved by the program to calculate the IGFBP-3 concentration in the sample:

$0.771 = 0.00001x^3 - 0.0025x^2 + 0.1351x$

$x = 6.617$

If the dilution factor (1:505) is taken into account the IGFBP-3 concentration of the undiluted sample is

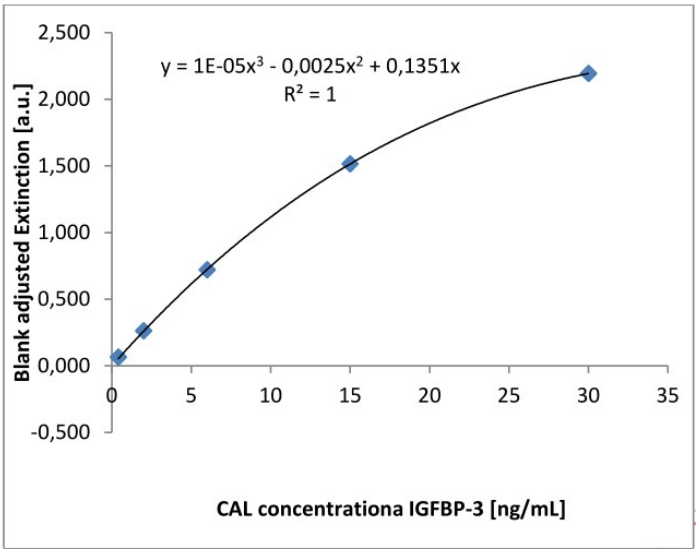
6.617 × 505 = 3342 ng/mL

Typical Standard Curve

The following data is for demonstration only and cannot be used in place of data generation at the time of assay.

	Blank	A	B	C	D	E
ng/mL	0	0.4	2	6	15	30
$OD_{(450\text{-}620\ nm)}$	0.204	0.254	0.453	0.911	1.706	2.390

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



Precision

Intra-Assay Variance

One sample has been measured 10 times in the same assay. The measured coefficient of variation (CV) is on average 1.9%.

	Determinations [n]	Mean value [ng/mL]	SD	CV [%]
Sample 1	10	3630	70.83	1.95
Sample 2	10	3789	83.75	2.21
Sample 3	10	3016	46.71	1.55

Inter-Assay-Variability

Serum samples were measured in independent assays on different days. On average the coefficient of variation was 5.7%.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
Mean [ng/mL]	2886	3525	3229	3219	4025	3293	3889	4328
SD	193	178	140	237	171	177	199	322
CV [%]	6.68	5.05	4.34	7.36	4.25	5.38	5.12	7.44
n	4	10	9	7	10	10	7	10

Sensitivity

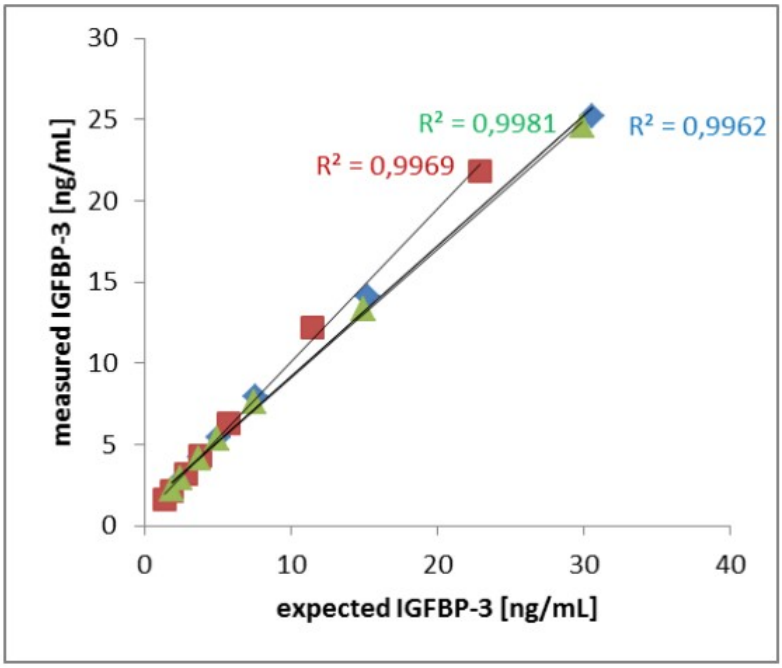
Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the 2fold standard deviation of the blank. The analytical sensitivity of Human IGFBP-3 ELISA Kit is 0.03 ng/mL. According ICH Q2 R1 (CPMP/ICH/381/95) the limit of quantification (LoQ) is reflected by the recalculated IGFBP-3 concentration of the 10fold standard deviation of the blank, which therewith is 0.15 ng/mL.

Specificity

The cross-reactivity of the antibodies used for Human IGFBP-3 ELISA Kit to homologous proteins was evaluated by diluting IGFBP-1, -2,-4,-5 and -6 in assay buffer to a concentration of 200 ng/mL and subsequent measurement of IGFBP-3. The relative cross-reactivities were ≤ 0.125%.

Linearity

Linearity was proven by dilution of three different serum samples with known IGFBP-3 concentration. The IGFBP-3 concentration of the diluted sample was measured and compared with the concentration expected. None of IGFBP-3 concentrations of the dilutions (1:125 to 1:2000) deviated more than 20% of the expected value (≤ -17%).



Recovery

Serum and plasma samples were enriched with recombinant IGFBP-3 and the recovery was calculated in comparison to buffer enriched with the same amount of IGFBP-3. The native samples used had an IGFBP-3 concentration of 2684 to 3667 ng/mL and the relative recovery was 109 – 118%.

IGFBP-3		Sample [ng/mL]	Sample enriched [ng/mL]	Target value [ng/mL]	Recovery [%]
Sample 1	Plasma	3641	5107	4324	118
Sample 2	Plasma	3667	4778	4350	110
Sample 3	Serum	2869	3778	3552	106
Sample 4	Serum	2684	3677	3367	109

Interferences

Interference of physiological appearing substance with the IGFBP-3 measurement was investigated. Serum samples have been enriched with different concentrations of possibly interfering substances and the amount of IGFBP-3 was measured and compared with the IGFBP-3 concentration in the same sample without any enrichment. None of the tested substances interfered significantly with IGFBP-3 measurement.

	Triglyceride (100 mg/mL)	Bilirubin (100 µg/mL)	Hemoglobin (5 mg/mL)
Sample 1	89	93	81
Sample 2	87	91	106
Sample 3	88	96	93

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Creative Diagnostics will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.
3. Do not use obviously damaged or microbial contaminated or spilled material.
4. This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.
5. Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.
6. Following components contain human serum: Controls CTR1 / CTR2, Calibrators A-E.

   Source human serum for the Controls provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.

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 craniopharyngioma, 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. The Human IGFBP-3 ELISA Kit is based on polyclonal rabbit antibodies. Generally, this technique is sensible to heterophilic antibodies in the sample. The influence of heterophilic antibodies is reduced by assay design, but cannot be excluded completely.