Version 14-11/24

User's Manual

Human IGFBP-6 (insulin-like growth factor binding protein 6) ELISA Kit

REF **DEIA-NB24-16**

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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.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

This enzyme immunoassay kit is suited for measuring IGFBP-6 in human serum / plasma and other body fluids. For research use only. Not for diagnostic procedures.

General Description

The Insulin-like Growth Factor 6 (IGFBP-6) is part of the IGF-System which consists of several binding proteins (1-6) and IGF-I and –II. The Insulin-like Growth Factors are involved in the control of human growth and regulated themselves by their binding proteins. The IGFBPs show high amino acid sequence homology, they have conserved N- and C-terminal domains which are involved in IGF-binding. In contrast the central linker region is highly variable. This region is not directly involved in IGF-binding but influences stability and localisation of the IGFBP / IGF complex and can be modified by glycosylation and phosphorylation.

IGFBP-6 a protein of 213 amino acids and about 34 kDa is somewhat special because of only three disulphide bridges in the C-terminal domain, resulting in a significantly higher affinity (50fold) for IGF-II than IGF-I. Further IGFBP-6 can be O-glycosylated and is cleaved by cathepsin-D-like acid protease, neutral serine protease as well as MMP-2/-7/-9/-12.

Main proposed function of IGFBP-6 is the regulation of the biological availability of IGF-II and thereby it influences cell proliferation, differentiation, migration and survival. Further IGFindependent as well as intra- and nuclear actions of IGFBP-6 are discussed. IGFindependent actions might be transmitted by prohibitin-2 as a potential cell surface receptor. Physiologically IGFBP-6 might be involved in senescence, angiogenesis and cancer. But a clear clinical indication for measurement of plasma IGFBP-6 needs to be determined.

Principles of Testing

The Human IGFBP-6 ELISA Kit is a so-called Sandwich-Assay. It utilizes two specific antibodies of high affinity. First the IGFBP-6 in the sample binds to the immobilized antibody on the microtiter plate. In a two-step sequence, the biotin-conjugated anti-IGFBP-6-Antibody and the streptavidin-peroxidase are bound. Subsequently, the peroxidase catalyzes an enzymatic reaction resulting in a blue coloration. The intensity of the blue color depends on the analyte content of the sample. The reaction is stopped by the addition of stop solution and color intensity (yellow) is quantified by measuring the absorption.

Human IGFBP-6 ELISA Kit allows secure and reproducible measurement of IGFBP-6 in human body fluids and is a suitable tool for the investigation of IGFBP-6 as a biomarker in growth, cancer and bone metabolism. In a preliminary study IGFBP-6 was measured in serum of healthy blood donors and mean concentration of 204 ng/mL was detected (Range: 73-367, n=20).

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with rabbit-anti-hIGFBP-6-antibody. Wells are separately breakable. (8x12) wells
2. **Standards (A-E):** lyophilized, (recombinant mouse IGFBP-6), concentrations are given on vial labels and on quality certificate. 5 x 750 µL

3. **Control Serum 1 (KS1):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 250 µL
4. **Control Serum 2 (KS2):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 250 µL
5. **Antibody Conjugate (AK):** ready for use, contains rabbit biotinylated anti-hIGFBP-6 antibody. 1 x 12 mL
6. **Enzyme Conjugate (EK):** contains HRP (Horseradish-Peroxidase)-labeled Streptavidin. 1 x 12 mL
7. **Dilution Buffer (VP):** ready for use. Please shake before use! 1 x 120 mL
8. **Washing Buffer (WP):** 20-fold concentrated solution. 1 x 50 mL
9. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised Tetramethylbencidine. 1 x 12 mL
10. **Stopping Solution (SL):** 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 standards A-E and Control Sera KS1 and KS2 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 samples as well as other body fluids and cell culture medium

The influences of anti-coagulants on IGFBP-6 measurements by Human IGFBP-6 ELISA Kit were investigated in corresponding EDTA and serum samples. One of 10 samples showed a significant reduction of IGFBP-6 concentration in EDTA/Aprotinin Plasma compared with serum of ~40%. On average recovery of serum IGFBP-6 in EDTA (n=10) and heparin (n=5) plasma was 102 and 98%, respectively. Additionally, recovery of recombinant IGFBP-6 was evaluated in several body fluids (breast milk, saliva, amniotic fluid,

urine) as well as in RPMI cell culture medium was tested. The recovery was not impaired in any tested fluids except urine.

2. Specimen collection

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

3. Required sample volume: 10 µL

4. Sample stability

In firmly closable sample vials

• Storage at 20-25°C: max. 2 days

• Storage at -20° C: min. 1 year

• Freeze-thaw cycles: max. 1 (sample dependent an increase in signal intensity was detected after more cycles)

It is recommended to keep sample refrigerated or frozen as soon as possible after separation of coagulated and corpuscular blood components and to avoid more than 1 freeze-thaw cycles.

5. Interference

Generally, haemolytic, icteric and lipemic samples should be avoided. Interference was tested by addition of triglycerides, haemoglobin or bilirubin to serum samples and measurement of IGFBP-6 recovery. At 100 mg/mL, 5 mg/mL and 200 µg/mL no significant (<30%) interference was detected.

6. Sample dilution

• Dilution: 1:51 with Dilution Buffer VP

• Pipette 500 µL Dilution Buffer VP in PE-/PP-Tube (application of a multi-stepper is recommended in larger series); add 10 µL sample (dilution 1:51). After mixing use 2 x 100 µL of this dilution in the assay.

• Attention: serum and plasma samples must be diluted at least 1:20 in Dilution Buffer VP.

• Depending on the expected IGFBP-6 values the samples can be diluted higher in Dilution Buffer VP.

• 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 Standards A – E is reconstituted with 750 µL Dilution Buffer VP. After resuspension, the standards are diluted according to a gradient - A (0.1 ng/mL), B (0.5 ng/mL), C (1 ng/mL), D (5 ng/mL), E (10 ng/mL), which are prepared for immediate use.
2. The Control KS1 and KS2 are reconstituted with 250 µL Dilution Buffer VP. After reconstitution dilute the Control KS with the Dilution Buffer VP in the same ratio (1:51) 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 WP 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, Standards A-E, Control Serum KS1/2 and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody Conjugate AK, Enzyme Conjugate EK 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 SL should be added to the plate in the same order as Substrate S. All determinations (Blank, Standards A-E, Control Serum KS1/2 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. Washing: 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 WP diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least.

   Manual washing: Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. Decant contents into a biohazard bin and then blot plate on absorbent tissue. Wash the plate by adding 300 µL Washing Buffer WP/well, then decant and blot on absorbent tissue. Repeat this step 4 more times for total of 5 washes.

Assay Step

1. Set Standards A-E, test samples (1:51 diluted), Control Serum KS1/2 (1:51 diluted) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate.
2. Aliquot 100ul of Dilution Buffer VP (Blank), Standards A-E, Control Serum KS1/2 and test samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 20-25°C, 350 rpm for 1 hour.
4. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
5. Add 100ul Antibody Conjugate AK 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 1 hour.
6. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
7. Add 100ul Enzyme Conjugate EK 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 30 minutes.
8. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not

let the wells dry completely at any time.

9. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark for 30 minutes.
10. Stop: Add 100ul Stopping Solution SL into each well.
11. 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.

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 standard E should be above 1.00. Samples, which yield higher absorbance values than Standard E, should be re-tested with a higher dilution.

Standard A (0.1 ng/mL) should be within +/-25% of its nominal value and ≤ 25% CV.

Standards B – E (0.5-10 ng/mL) should be within +/-20% of their nominal values and ≤ 20% CV.

Controls KS1 and KS2 should be ≤ 20% CV and be within the specified range.

The assay is valid when both control sera KS1 and KS2 as well as 5/6 standards met the acceptance criteria specified.

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 standards.
3. Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
4. 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 four parametric logistic (4-PL) curve fit should be used for recalculation of IGFBP-6 concentrations.
5. The IGFBP-6 concentration in ng/mL of the samples and controls KS1 and KS2 can be calculated by multiplication with the dilution factor of 1:51.

Exemplary calculation of IGFBP-6 concentrations

Sample dilution: 1:51

Measured extinction of your sample 1.495

Measured extinction of the blank 0.055

Your measurement program will calculate the IGFBP-6 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.

In this exemplary case the following equation is solved by the program to calculate the IGFBP-6 concentration in the sample:

$y = -0.0125x^2 + 0.3724x$

$x = 1.44$

If the dilution factor (1:51) is taken into account the IGFBP-6 concentration of the undiluted sample is

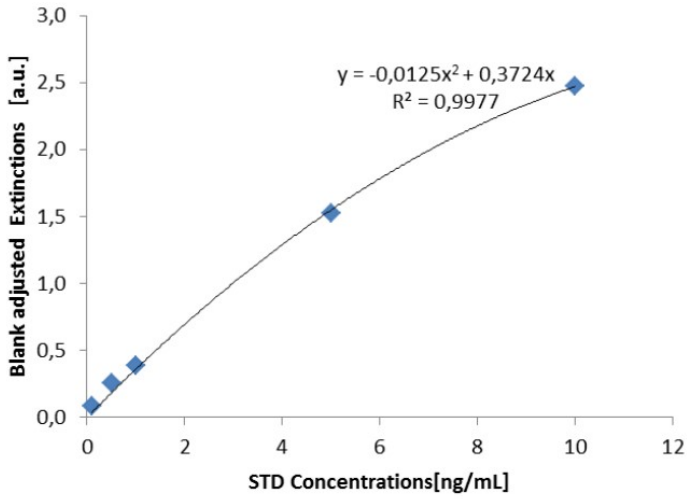
$1.44 \times 51 = 73.44$ ng/mL

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	0.1	0.5	1	5	10
$OD_{(450\text{-}620\ nm)}$	0.06	0.08	0.26	0.39	1.52	2.48

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!



Reference Values

IGFBP-6 was measured in blood samples of healthy blood donors. Measured mean is 204 ng/mL (Range: 73 – 367).

Precision

Intra-Assay Variance

IGFBP-6 content of four samples was measured at least 12 times in the same assay. The measured coefficient of variation (CV) is 1.1% on average.

Inter-Assay Variance

IGFBP-6 content of 12 serum samples was measured in 11 or more independent assays. The measured coefficient of variation (CV) is 6.67% on average.

Sensitivity

Sensitivity was assessed by measuring the signal of the blank at least 8 times within one test and calculating the theoretical concentration of 2SD of the mean signal measured. The analytical sensitivity of the Human IGFBP-6 ELISA Kit is on average 0.026 ng/mL, measured in 4 independent tests (Range 0.004 to 0.029 ng/mL).

Based on the minimal required dilution (1:20) and lowest calibration standard (0.1 ng/mL) the lower limit of quantification is 2 ng/mL in the undiluted sample.

Specificity

Influence of IGFs and IGFBP-2 to -5 on IGFBP-6 measurement was evaluated by direct measurement of potentially cross reactive substances in dilution buffer as well as on IGFBP-6 recovery in standard preparations.

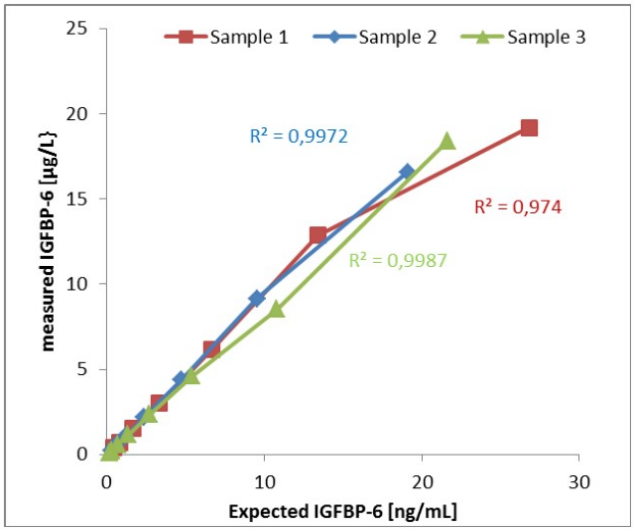
	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGF-I	IGF-II
[ng/mL]	**250**	**600**	**250**	**250**	**500**	**500**
Cross reactivity in VP [%]	0	0.1	0	0	0	0
Recovery of STD E [%]	0	5	-3	-7	-5	-17
[ng/mL]	**50**	**300**	**50**	**50**	**100**	**100**
Cross reactivity in VP [%]	0.05	0.08	0.05	0.02	0	0.03
Recovery of STD E [%]	-3	2	-4	-9	-10	-9

Cross reactions with animal samples

Several commercially available animal sera have been used as samples in the Human IGFBP-6 ELISA Kit in a dilution of 1:51 no significant signal (< STD A) was detected. Thus, this assay cannot be used for IGFBP-6 measurement in serum samples of cat, cattle, chicken, dog, donkey, goat, guinea pig, horse, mouse, pig, rabbit, rat and sheep.

Linearity

Linearity of the Human IGFBP-6 ELISA Kit was tested by dilution (1:5 up to 1:640) of native sera with different IGFBP-6 contents (Sample 1-3). A comparison of expected and measured concentrations is shown in Figure below. It becomes apparent that a dilution of 1:10 is not sufficient to allow linearity in all samples. These results were confirmed according to NCCLS/CLSI EP6-A 2003 standard by statistical analysis. A minimal dilution of ≥ 1:20 is required for linearity. The linearity of sample dilution is acceptable within the standard curve range.



Recovery

Recombinant IGFBP-6 was added in different amounts to human serum. The IGFBP-6 content of the so enriched samples was measured and recovery in comparison to enriched buffer calculated.

rec. IGFBP-6 [ng/mL]	Recovery as relative expected value [%]		
	Sample 1	Sample 2	Sample 3
272	94.98	94.50	95.59
4.573	101.73	102.36	103.81

Interferences

Interference of haemoglobin, bilirubin and triglycerides was tested by adding different amounts of these substances to human serum containing IGFBP-6. For comparison the same amount of buffer without any substance was also added to the serum. Table below demonstrates that neither haemoglobin, bilirubin nor triglycerides exert significant influence on the measurement of IGFBP-6 in human serum.

	Triglyceride (100 mg/mL)	Bilirubin (200 µg/mL)	Hemoglobin (5 mg/mL)
Sample 1	99	96	68
Sample 2	96	100	74
Sample 3	108	107	81

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: Control Serum KS1 and KS2

   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-6 levels increase with age and are higher in men than in women, serum levels decrease during pregnancy and increase in renal failure. In vitro IGFBP-6 expression is influenced by cAMP, IGFs, retinoic acid, Vitamin D, p53 and glucocortioids depending on the investigated cell model. TNF-β and β-catenin inhibit IGFBP-6 promoter activity (Bach, 2015). The Human IGFBP-6 ELISA Kit is based on polyclonal antibodies from rabbit. Generally, immunological assays are sensible to heterophilic antibodies and rheumatoid factors in the sample. Their influence is reduced by the assay design, but cannot be excluded completely.