Version 07-11/24

User's Manual

Mouse/Rat IGFBP-3 (insulin-like growth factor binding protein 3) ELISA Kit

REF **DEIA-NB24-15**

Σ **96T**

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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

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

This enzyme immunoassay kit is suited for measuring IGFBP-3 in mouse and rat serum and plasma and in cell culture medium.

General Description

Growth Hormone, Insulin-like Growth Factors and their binding proteins build up an endocrine system regulating not only longitudinal growth in humans but also influencing a broad variety of other physiological and pathophysiological processes like energy metabolism or tumor growth. Most effects of Growth Hormone (GH) are exerted by Insulin-like Growth Factors (IGF) mainly produced by the liver but also locally by specific tissues. The effects of IGF are also regulated, specific binding proteins (IGFBP 1-7) regulate bioavailability of IGF. After proteolytic cleavage of the binding proteins IGF is set free and able to bind to its receptor. The autophosphorylation of this thyrosine kinase receptor activates intra cellular signalling cascades. Some of these IGFBPs not only regulate the availability of IGF but also exert IGF-independent effects on cell physiology.

IGFBP-3 is the most abundant IGFBP in circulation and therefore of special relevance in regulation of IGF effects. This is reflected by the indicative value of serum IGFBP-3 concentration in diagnostics of growth disturbances. Regulation is effected e.g. through nourishing situation; Different diets for example affect the IGFBP-3 concentration (Bielohuby et al, 2010). IGFBP-3 has also been shown to be able to induce apoptosis, promote tumor growth and inhibit cellular migration and metastasis dependent on tissue and tumor stage. Mouse / rat models for in vivo experiments are often used for studies of IGF-dependent and independent effects of IGFBP-3, particularly in the field of tumor research.

Principles of Testing

The Mouse/Rat IGFBP-3 ELISA Kit is a so-called sandwich-assay. It utilizes two different specific high affinity polyclonal antibodies for this protein. The IGFBP-3 in the samples binds quantitatively to the immobilized antibody. In the following step, the biotinylated antibody in turn binds IGFBP-3. After washing, a streptavidin-peroxidase-enzyme conjugate will be added, which will bind highly specific to the biotin of the antibody. 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 goat anti-mouse-IGFBP-3 antibody. Wells are separately breakable. (8x12) wells
2. **Standards (A-G):** lyophilized, (native mouse IGFBP-3), concentrations are given on vial labels and on quality certificate. 7 x 750 µL
3. **Control Serum 1 (KS1):** lyophilized, (mouse serum), concentration is given on quality certificate. 1 x 250 µL

4. **Control Serum 2 (KS2):** lyophilized, (mouse serum), concentration is given on quality certificate. 1 x 250 µL
5. **Antibody Conjugate (AK):** ready for use, goat-anti-mouse-IGFBP-3 antibody biotinylated. 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 125 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
8. Graduated cylinder for diluting Washing Buffer (WP)

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-G 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: Mouse and Rat Serum Plasma.

In Heparin-Plasma samples the levels were found approx. 15% decreased. Further, cell culture medium was found to be suitable.

2. Specimen collection

Haemolytic reactions have to be avoided.

3. Required sample volume: 10 µL

4. Sample stability

In firmly closable sample vials

• Storage at -20°C: min. 2 years

• Freeze/-thaw cycles: max. 3

It is recommended to store samples as soon as possible at least at 4°C. For any long time storage the sample has to be kept frozen at -20°C.

5. Sample dilution

• Dilution: 1:505 with Dilution Buffer VP

• We recommend a dilution in 2 steps: Pipette 1 mL Dilution Buffer VP 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. Pipette 100 µL of this dilution into another PE/PP vessel with 400 µL of Dilution Buffer VP and mix immediately. This results in a final dilution of 1:505. After mixing, use 100 µL per assay in the assay within 1 hour of this solution.

• Where required, depending on the expected IGFBP-3-values, the dilution with Dilution Buffer VP can be higher or lower.

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 – G is reconstituted with 750 µL Dilution Buffer VP. After resuspension, the standards are diluted according to a gradient - A (0.39 ng/mL), B (0.78 ng/mL), C (1.56 ng/mL), D (3.13 ng/mL), E (6.25 ng/mL), F (12.5 ng/mL), G (25 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 (1:505) 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 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 H_2O_2 Tetramethylbencidine, is photosensitive–store and incubation in the dark.
2. When performing the assay, Blank, Standards A-G, Control Serum KS 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-G, Control Serum KS 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.

   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 Standards A-G, test samples (1:505 diluted), Control Serum KS1/2 (1:505 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-G, 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 15 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 15 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

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 G should be above 1.00. Samples, which yield higher absorbance values than Standard G, are beyond the standard curve, for reliable determinations such samples should be retested at 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 values.
3. Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
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 5.760 ng/mL, Sample was 1:505 diluted: 5.760 x 505= 2909 ng/mL, or 2.909 µg/mL equal to 2.909 mg/L)

Exemplary calculation of IGFBP-3 concentrations

Sample dilution: 1:505

Measured extinction of your sample 0.749

Measured extinction of the blank 0.000

Your measurement program will calculate the IGFBP-3 concentration of the 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:

$$0.749 = 3.62^{-6}x^3 - 0.00188x^2 + 0.143x - 0.011$$

$$x = 5.760$$

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

$$5.760 \times 505 = 2909 \text{ ng/mL}$$

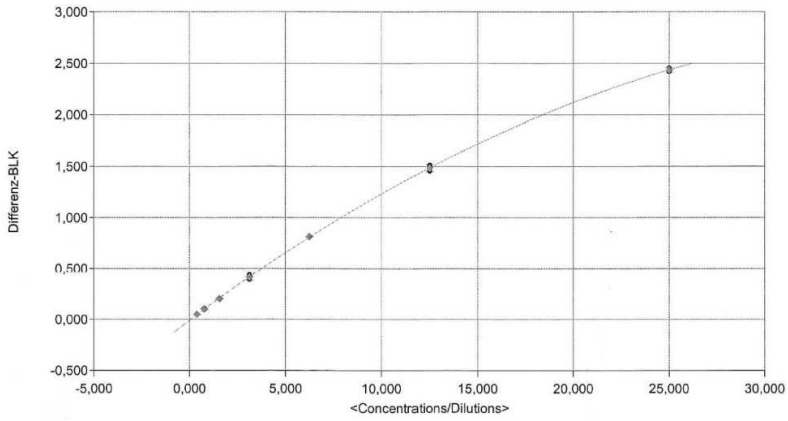
Typical Standard Curve

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	F	G
ng/mL	0	0.39	0.78	1.56	3.13	6.25	12.5	25
$OD_{(450-620\ nm)}$	0.00	0.048	0.101	0.202	0.412	0.815	1.484	2.438

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

The Inter- and Intra-Assay variation coefficients were on average ≤10%.

Intra-Assay-Variation (n=13)

	Mean value [ng/mL]	SD	CV [%]
Sample 1	3286	124	3.76
Sample 2	1529	123	8.02

Inter-Assay-Variation (n=26 or 15)

	Mean value [ng/mL]	SD	CV [%]
Sample 1	4836	384	7.94
Sample 2	2625	269	9.43

Sensitivity

The analytical Sensitivity was assessed by 21-fold determination of the blank and calculating the theoretical concentration of the blank +2SD. The analytical sensitivity of the Mouse/Rat IGFBP-3 ELISA Kit is 0.09 ng/mL.

Specificity

No cross reactivity was detected for: Rabbit, Cat, Chicken, Guinea pig, Goat, Sheep, Horse, Donkey, Pig, Dog, Bovine.

Cross reactivity with recombinant human eukaryotic expressed IGFBP-3 (1 µg/mL): 0.06%

Linearity

Dilution	Sample 1 [ng/ml]	Sample 2 [ng/ml]
1:100	3518	3676
1:200	3691	4145
1:400	3845	4234
1:800	3813	4110
1:1600	3792	4219
1:3200	3861	4557
AV / SD / CV%	**3753/ 129/ 3.46**	**4157/ 284/ 6.83**

Recovery

The recovery of recombinant mouse IGFBP-3 in cell culture medium DMEM was found to be 89.4%, and, in DMEM incl. 5% FCS 92.6%. Therefore, cell culture medium seems to be suitable as sample matrix.

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.