Version 07-11/24

User's Manual

Human Growth Hormone Binding Protein (GHBP) ELISA Kit

REF **DEIA-NB24-05**

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.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Human GHBP ELISA Kit is intended to be used for quantitative measurement of GHBP in human serum and plasma samples.

General Description

Growth Hormone Binding Protein (GHBP) consists of 238 amino acids and includes four sides for glycosylation and three disulphide bounds. In humans GHBP is formed by receptor shedding of the growth hormone receptor by a metalloprotease (ADAM17). In equilibrium about 50% of circulating growth hormone (GH) is bound to GHBP but only 2% of the circulating GHBP bound a GH molecule with a stoichiometry of 1:1. Only in case of supraphysiological GHBP levels a 2:1 ratio appears. The complex of GH and GHBP has an approximate molecular weight of 80 kDa (GHBP 60 kDa). In an animal model (guinea pig) the complex formation increases half-life from 11-20 minutes up to about 100 minutes and in general binding to GHBP inhibits GH cellular action.

GHBP Physiology:

GHBP concentration is independent of GH pulsatility and does not show a circadian rhythm. GHBP levels are low until 2-6 months of life, increase steeply in the first two years and continue to increase slowly until early adulthood. From the 4th decade the GHBP serum concentration declines slowly. GHBP correlates positively with the intraabdominal fat mass and is increased in type II diabetics with hyperinsulinemia. It is not known whether the tight relationship between fat mass and circulating GHBP results from GHBP expression in adipocytes or any other mechanism. From a scientific point of view undetectable GHBP levels could point to a GH insensitivity, caused by a deletion in the GH-receptor gene. Further, the IGF-I/GHBP ratio might be an indicator for GH-deficiency in adults, in particular in women. It could also be predictive for GH treatment response. The strong positive relationship with intraabdominal fat mass might be a hint, that GHBP is a possible biomarker for the amount of visceral adipose tissue.

Principles of Testing

The Human GHBP ELISA Kit is based on polyclonal antibodies and recombinant GHBP, expressed in eukaryotic cells. The Human GHBP ELISA Kit is a so-called Sandwich-Assay. It utilizes two specific antibodies of high affinity. First the GHBP in the sample binds to the immobilized antibody on the microtiter plate. In a two-step sequence, the biotin-conjugated anti-GHBP-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 GHBP content of the sample. The reaction is stopped by the addition of stop solution and color intensity is quantified by measuring the absorption. The Human GHBP ELISA Kit allows secure and reproducible measurement of GHBP in human body fluids and is a suitable tool for the investigation of GHBP as biomarker in energy and fat metabolism. In a preliminary study GHBP was measured in serum of healthy blood donors and mean concentration of 16.28 ng/mL was detected (Range: 12.48 - 22.31).

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with rabbit-anti-GHBP-antibody. Wells are separately breakable. (8x12) wells
2. **Sample Buffer (PP):** ready for use, please shake before use! 1 x 120 mL
3. **Standards (A-F):** lyophilized, (recombinant GHBP in rabbit serum), concentrations are given on vial labels and on quality certificate. 6 x 750 µL
4. **Control Serum 1 (KS1):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 250 µL
5. **Control Serum 2 (KS2):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 250 µL
6. **Antibody Conjugate (AK):** ready for use, contains rabbit biotinylated anti-GHBP antibody. 1 x 12 mL
7. **Enzyme Conjugate (EK):** ready for use, contains Streptavidin-Peroxidase Conjugate. 1 x 12 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. 3

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 at 2-8°C, 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-F 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

Serum and Heparin/EDTA plasma yield comparable values. The GHBP levels are reduced in citrate plasma samples, because of the relatively high amount of anticoagulant.

2. Specimen collection

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

3. Required sample volume: 15 µL

4. Sample stability

In firmly closable sample vials

• Storage at 4°C: max. 3 days

• Freezer /-thaw cycles: max. 3

Freeze-thaw cycles should be minimized. Up to 3 cycles showed no effect on the measured GHBP concentration. First experiments with native serum samples were conducted, incubating these samples at 20-25°C and 27°C for three days. A significant decay of GHBP was detected in the samples incubated at 37°C (>20%). The decrease in GHBP at ambient temperature was less prominent (-8 to -13%) and at 4°C no significant change was detected.

5. Interference

Neither triglycerides, bilirubin nor hemoglobin exert any influence up to concentrations of 100 g/L, 200 mg/L, 5 g/L respectively on the measurement of GHBP in human serum.

6. Sample dilution

• Dilution: 1:21 with Sample Buffer PP

• Example: 15 µL sample to 300 µL sample buffer PP provided (21 dilution factor).

7. GHBP in healthy adults

Exemplary GHBP was measured in healthy human blood donors (n=10). The mean GHBP concentration detected was 16.28 ng/mL (Range: 12.48 to 22.31)

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 – F is reconstituted with 750 µL Sample Buffer PP. After resuspension, the standard is diluted according to a gradient - A (0.125 ng/mL), B (0.25 ng/mL), C (0.5 ng/mL), D (1.0 ng/mL), E (2.0 ng/mL) and F (4.0 ng/mL), which are prepared for immediate use.
2. The Control KS1 and KS2 are reconstituted with 250 µL Sample Buffer PP. After reconstitution dilute the Controls KS1 and KS2 with the Sample Buffer PP in the same ratio (1:21) 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

When performing the assay, Blank, Standards A-F, Controls KS1, KS2 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 Solution S should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution SL should be added to the plate in the same order as Substrate Solution S. All determinations (Blank, Standards A-F, Controls KS1, KS2 and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

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

Shaking-The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must 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.

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. The danger of handling with potentially infectious material must be taken into account.

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

Manual washing is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by 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 Blank, Standard A-F, test samples (1:21 diluted), Control Serum KS1 and KS2 (1:21 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. Prepare Standards: Aliquot 100ul of Sample Buffer PP as Blank, Standard A-F, Control Serum KS1/KS2 or 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 within 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

GLP requires that controls must 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. All 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.

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 F should be above 1.00. Samples, which yield higher absorbance values than Standard F, 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 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. 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 GHBP concentration in ng/mL (or pg/mL, according the chosen unit for the standards) of the samples can be calculated by multiplication with the respective dilution factor Quality Criteria.

Exemplary calculation of ALS concentrations

Sample dilution: 1:21

Measured extinction of your sample.....0.694

Measured extinction of the blank.....0.084

Your measurement program will calculate the GHBP 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 GHBP concentration in the sample:

$0.610 = -0.0142x^2 + 0.5971x$

X= 1.05 ng/mL

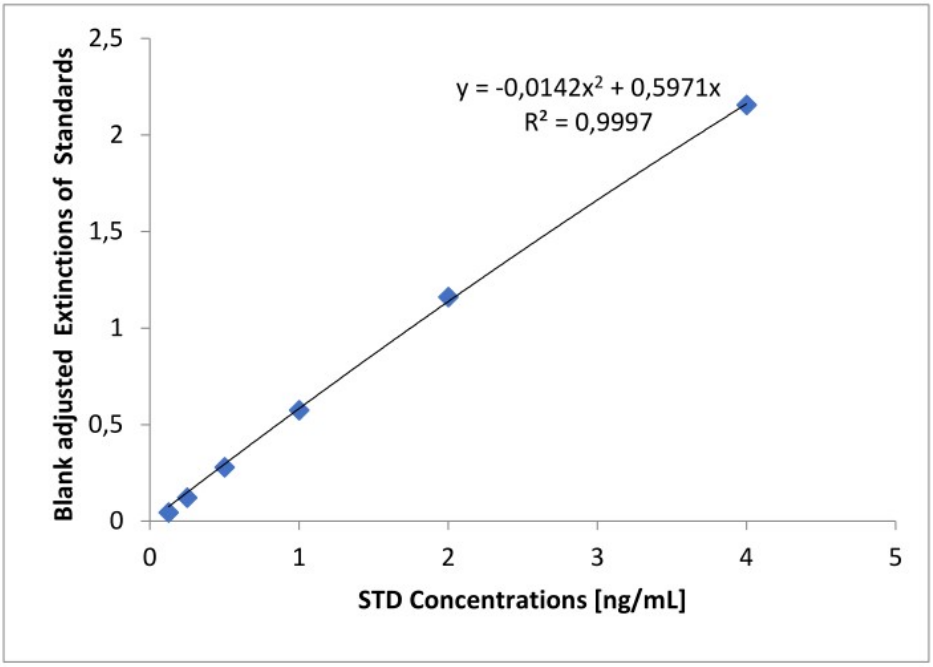
If the dilution factor (1:21) is taken into account the GHBP concentration of the undiluted sample is:

1.05 ng/mL x 21 = 21.96 ng/mL

Typical Standard Curve

The exemplary data and the standard curve in Figure below cannot be used for the calculation of the test results. You have to establish a standard curve for each test you conduct.

Standard	A	B	C	D	E	F
ng/mL	0. 125	0. 25	0.5	1.0	2.0	4.0
$OD_{450\text{-}620\ nm}$	0.045	0.122	0.279	0.575	1.160	2.156



Precision

Intra-Assay-Variation

A native serum sample has been measured 16 times on different positions on the plate and at a mean concentration of 14.89 ng/mL GHBP (SD 0.65) an intra-assay variability of 4.38% was detected.

Inter-Assay-Variation

Serum samples were measured in independent assays. On average the coefficient of variation was 7.72% (Range 3.08 – 10.67%).

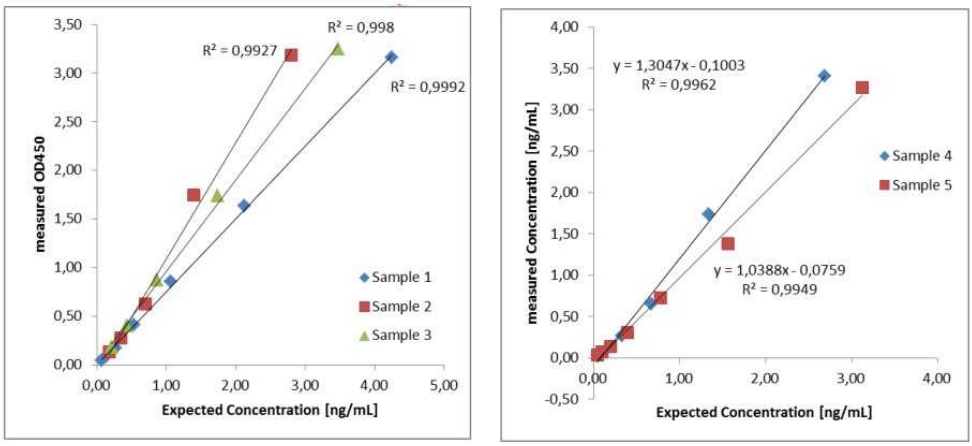
Sensitivity

The analytical sensitivity (LoD) was assessed by measuring the blank and calculating the theoretical

concentration of the blank + 2SD. The analytical sensitivity of the Human GHBP ELISA Kit is 0.009 ng/mL as mean, in 3 independent determinations values ranging from 0.007 to 0.010 ng/mL were found. The theoretical limit of quantification (LoQ) (10 times the standard deviation of the blank value) is 0.043 ng/mL.

Linearity

Linearity was tested by dilution of native sera with different GHBP contents (Sample 1-5). The optical density was measured and plotted against the expected GHBP concentration. Linearity was analysed by linear regression, a coefficient of correlation >0.9 indicates a good linearity.



A closure look to the data revealed that a dilution of 1:10 is possible but good linearity is realized from a dilution of 1:20 in sample buffer. Here the deviation of the mean is less than 30%.

Recovery

1 ng/mL recombinant GHBP was added to human serum. The GHBP content of the so enriched samples was measured and recovery calculated. Results are shown in table below.

[µg/L]	Sample 1	Sample 2	Sample 3
Sample	17.73	16.61	10.07
1 ng/mL + GHBP	18.81	17.28	12.51
% Recovery	100	98	113

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 Sera KS1 and KS2, and Standards A-F. Source human serum for the control sera 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

The Human GHBP ELISA Kit is based on polyclonal antibodies. The measurement results determined by this technique can be influenced by heterophilic antibodies. The potential influence of these antibodies was minimized by assay design but can never be excluded completely. Further, several physiological substances like triglycerides were tested regarding their influence on GHBP measurement and no significant influence was detected. But in theory there might be other substances or other concentration which interfere with GHBP measurement.