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GHBP-ELISA

Enzyme Immunoassay for the Measurement of

Growth Hormone Binding Protein

Concentrations

For Research Use Only - Not for use in diagnostic procedures.

Storage at: 2-8°C Kitsize: 96

Cat.-No.: E024

Gesellschaft für Forschung und Herstellung von Diagnostika GmbH

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Manufactured by Mediagnost / Germany
for IBL-America Minneapolis MN55432

Table of Contents

Instructions for use summary

GHBP ELISA E024	96 Determinations
Regulatory Status	For Research Use Only. Not for diagnostic purposes.
Principle of the test	Sandwich Enzyme immunoassay
Duration (incubation period)	3 h
Antibodies	specific, high-affinity polyclonal antibodies
Buffer	Ready for use
Reference material	eukaryotic, recombinant GHBP
Standard	6 single standards: 0.05 – 4ng/mL, recombinant GHBP
Assay Range	0.006 – 84 ng/mL
Control	2 control sera, freeze-dried
Sample	human serum / plasma
Required sample volume	15 µL
Sample dilution	1:21
Analytical sensitivity	0.006 ng/mL
Intra- / Interassay Variance	<10 / <10 %

1 INTENDED USE

Precise measurement of human Growth Hormone Binding Protein levels in serum or plasma. For research use only.

2 INTRODUCTION[1]

Growth Hormone Binding Protein (GHBP) consists of 238 amino acids and includes four sides for glycosylation and three disulfide 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 supra-physiological 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.

[1] Fisker S Physiology and pathophysiology of growth hormone-binding protein: Methodological and clinical aspects. Growth Hormone & IGF Research 2006: 16 (1-28)

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.

3 ASSAY PRINCIPLE

This ELISA assay coded E024 is based on polyclonal antibodies and recombinant GHBP, expressed in eukaryotic cells.

This ELISA for GHBP 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.

This GHBP ELISA, cat.-no. E024 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).

4 WARNINGS AND PRECAUTIONS

For research and professional use only.

The Mediagnost kit is suitable only for in vitro use and not for internal use in humans and animals. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Mediagnost 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.

Do not use obvious damaged or microbial contaminated or spilled material.

Caution: This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.

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.

Human Serum

Following components contain human serum: **Control Sera KS, KS2 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.

Reagents AK, EK, PP, WP

Contain as preservatives f **5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one** (<0.015%)

H317	May cause an allergic skin reaction.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P272	Contaminated work clothing should not be allowed out of the workplace.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P333+P313	If skin irritation or rash occurs: Get medical advice/ attention.
P302+P352	IF ON SKIN: Wash with plenty of soap and water.
P501	Dispose of contents/ container in accordance with local/ regional/ national/ international regulations.

Substrate Solution (S)

The TMB-Substrate (S) contains 3,3´,5,5´ Tetramethylbencidine (<0.05%)

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
P261	Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.

Stopping Solution (SL)

The Stopping solution contains 0.2 M sulfuric acid (H_2SO_4)

H290	May be corrosive to metals.
H314	Causes severe skin burns and eye damage.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P301+P330+	IF SWALLOWED: rinse mouth.
P331	Do NOT induce vomiting.
P305+P351+	IF IN EYES: Rinse cautiously with water for several minutes.
P338	Remove contact lenses, if present and easy to do. Continue rinsing.
P309+P310	IF exposed or if you feel unwell: Immediately call a POISON CENTER or doctor/physician.

4.1 General first aid procedures:

Skin contact: Wash affected area rinse immediately with plenty of water at least 15 minutes. Remove 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: After swallowing the product, if the affected person is conscious, rinse out the mouth with plenty of water: seek medical advice immediately.

5 SAMPLES

5.1 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.

5.2 Specimen collections

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

5.3 Required sample volume

15 µL

5.4 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.

5.5 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.

5.6 Sample dilution

- **Dilution: 1: 21** with sample buffer **PP**
- **Example: 15 µL** sample to **300 µL** sample buffer **PP** provided (21 dilution factor).

5.7 GHBP in healthy adults

Exemplary GHBP was measured in healthy human blood donors (n=10). The mean GHBP concentration detected was 16.28ng/mL (Range: 12.5 to 22.3)

6 MATERIALS

6.1 Materials Provided

The reagents listed below are sufficient for 96 wells including the standard curve.

MTP	**Microtiter plate,** ready for use, coated with rabbit-anti-GHBP-antibody. Wells are separately breakable.	**(8x12) wells**
A-F	**Standards**, lyophilized, (recombinant GHBP in rabbit serum), concentrations are given on vial labels and on the QC-certificate.	**6x 750 µL**
KS1	**Control Serum 1,** lyophilized, (human serum), concentration is given on the QC-certificate.	**1 x 250 µL**
KS2	**Control Serum 2,** lyophilized, (human serum), concentration is given on the QC-certificate.	**1 x 250 µL**
AK	**Antibody-Conjugate,** ready for use, contains rabbit biotinylated anti-GHBP antibody**.**	**1 x 12 mL**
EK	**Enzyme-Conjugate,** ready for use, contains Streptavidin-Peroxidase Conjugate**.**	**1 x 12 mL**
PP	**Sample Buffer,** ready for use, **Please shake before use!**	**1 x 120 mL**
WP	**Washing Buffer,** 20-fold concentrated solution	**1 x 50 mL**
S	**Substrate,** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised H_2O_2 Tetramethylbencidine.	**1 x 12 mL**
SL	**Stopping Solution,** ready for use, 0.2 M sulphuric acid.	**1 x 12 mL**
-	**Sealing Tape,** for covering the **microtiter plate.**	**3 x**
↓	**Instructions for use**	**1 x**
--	**Quality Control Certificate**	**1 x**

6.2 Materials required, but not provided

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

7 TECHNICAL NOTES

Storage Conditions
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.

Storage Life
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-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

Preparation of reagents
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.

Reconstitution
The Standards **A – F** and Controls **KS1** and **KS2** are reconstituted with the Sample Buffer **PP**. 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.

Dilution
After reconstitution dilute the Control Sera **KS1** and **KS2** with the Sample Buffer **PP** in the same ratio (1:21) as the sample.
The required volume of Washing Buffer **WP** is prepared by 1:20 dilution of the provided 20fold concentrate with Aqua dest.

Assay Procedure
When performing the assay, Blank, Standards **A-E**, Control Serum **KS1 and 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-HRP-Conjugate **AK** 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-E**, Control Sera **KS1 and 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 H2O2-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 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 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.

8 SUMMARY OF THE ASSAY PROCEDURE E024

Preparation of reagents		**Reconstitution:**	**Dilution**
A-F	**Standards**	in **750 µL** Sample Buffer **PP**	-
KS1	**Control Serum 1**	in **250 µL** Sample Buffer **PP**	**1:21** with **PP**
KS2	**Control Serum 2**	in **250 µL** Sample Buffer **PP**	**1:21** with **PP**
WP	**Washing Buffer**	-	**1:20** with **Aqua dest.**
Sample dilution: with Sample Buffer **PP 1:21. Don´t use samples undiluted!**			
Before assay procedure bring all reagents to room temperature **20-25°C**.			

Assay Procedure in Double Determination:

Pipette	**Reagents**	**Position**
100 µL	Sample Buffer **PP** as Blank	A1/A2
100 µL	Standard **A** (**0.05 ng/mL**)	B1/B2
100 µL	Standard **B** (**0.1 ng/mL**)	C1/C2
100 µL	Standard **C** (**0.5 ng/mL**)	D1/D2
100 µL	Standard **D** (**1 ng/mL**)	E1/E2
100 µL	Standard **E** (**1.5 ng/mL**)	F1/F2
100 µL	Standard **F** (**4 ng/mL**)	G1/G2
100 µL	Control Serum **KS 1** (1:21 diluted)	H1/H2
100 µL	Control Serum **KS 2** (1:21 diluted)	A3/A4
100 µL	Sample (1:21 diluted)	in the rest of the wells according the requirements
Cover the wells with the sealing tape.		
Sample Incubation: 1 h at 20-25°C, 350 rpm		
5 x 300 µL	Aspirate the contents of the wells and wash **5 x** with **300 µL** each Washing Buffer **WP/ well**	In each well
100 µL	Antibody -Conjugate **AK**	In each well
Cover the wells with the sealing tape.		
Incubation: 1 hour at 20-25°C, 350 rpm		
5 x 300 µL	Aspirate the contents of the wells and wash **5 x** with **300 µL** each Washing Buffer **WP/ well**	In each well
100 µL	Enzyme -Conjugate **EK**	In each well
Cover the wells with the sealing tape.		
Incubation: 30 minutes at 20-25°C, 350 rpm		
5 x 300 µL	Aspirate the contents of the wells and wash **5 x** with **300 µL** each Washing Buffer **WP/ well**	In each well
100 µL	Substrate Solution **S**	In each well
Incubation: 30 Minutes in the Dark at 20-25°C		
100 µL	Stopping Solution **SL**	In each well
	Measure the absorbance within 30 min at **450 nm** with ≥ 590 nm as reference wavelength.	

9 QUALITY CONTROL

GLP 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. 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.

10 EVALUATION OF RESULTS

10.1 Establishing of the Standard Curve

The standards provided contain the following concentrations of GHBP :

Standard	**A**	**B**	**C**	**D**	**E**	F
ng/mL	0.05	0.1	0.5	1	1.5	4

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

10.2 Example of Typical Standard Curve

The exemplary data and the standard curve in Figure 1 cannot be used for the calculation of the test results. You have to establish a standard curve for each test you conduct.

Table :1 Messdaten, die eine typische Standardkurve beschreiben.

Standard	**A**	**B**	**C**	**D**	**E**	**E**
ng/mL	0.05	0.1	0.5	1	1.5	4
$OD_{450-620}$	0.055	0.105	0.470	0.924	1.294	2.909

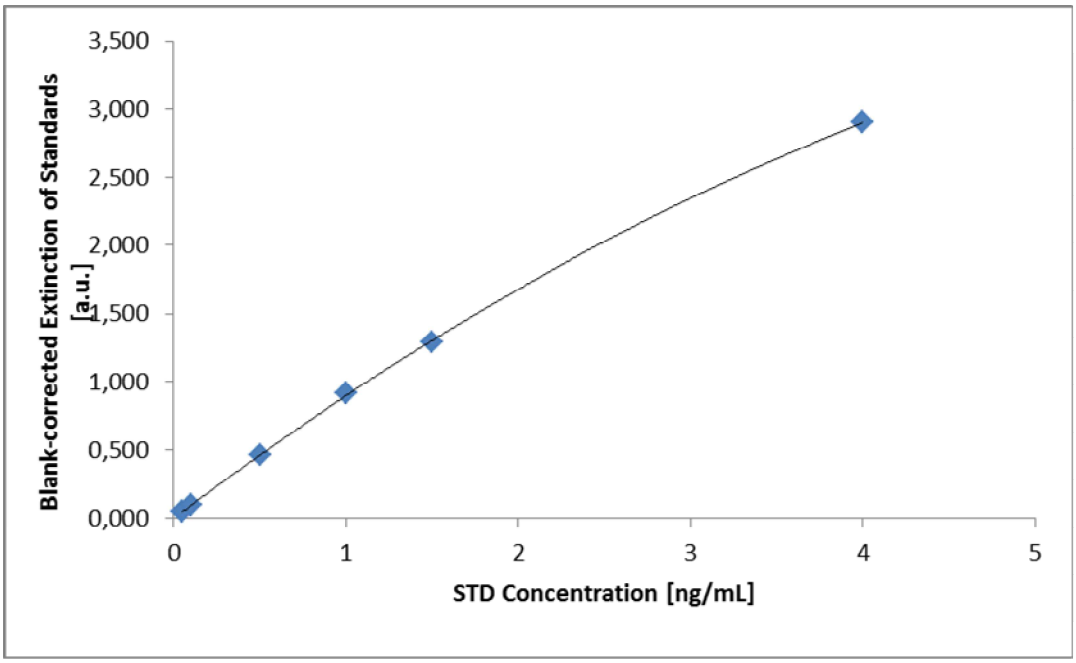

Figure 1: Exemplary Standard Curve

10.3 Evaluation of sample concentrations

Sample dilution: 1:21

Measured extinction of your sample: 0.624
Measured extinction of the blank: 0.059

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:

$0.565 = -0.0592x^2 + 0.964x$
X= 0.609 ng/mL

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

0.609 ng/mL x 21 = 12.798 ng/mL / µg/L

11 LIMITATIONS OF PROCEDURE

The Mediagnost GHBP ELISA E024 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.

12 PERFORMANCE CHARACTERISTICS

12.1 Sensitivity

Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the blank + 2SD. The analytical sensitivity of the Mediagnost E024 is 0.006 ng/mL as mean, in 3 independent determinations values ranging from 0.007 to 0.005 ng/mL were found.
Sensitivity was assessed further by determination of serially dilutions of native human serum samples (see Linearity). In these experiments samples where diluted up to 1:320 and GHBP measured in the dilution was 0.03 ng/mL. Theoretically, defined as the 10 standard deviations of the blank the limit of quantification is also 0.03 ng/mL GHBP.

12.2 Specificity

The specificity of the antibodies used for GHBP detection in the Mediagnost GHBP ELISA E024 was evaluated by seize exclusion chromatography analysis of human serum enriched with recombinant growth hormone (Fig 2) and subsequent analysis of SEC fractions by Mediagnost E022 or GHBP antibodies.

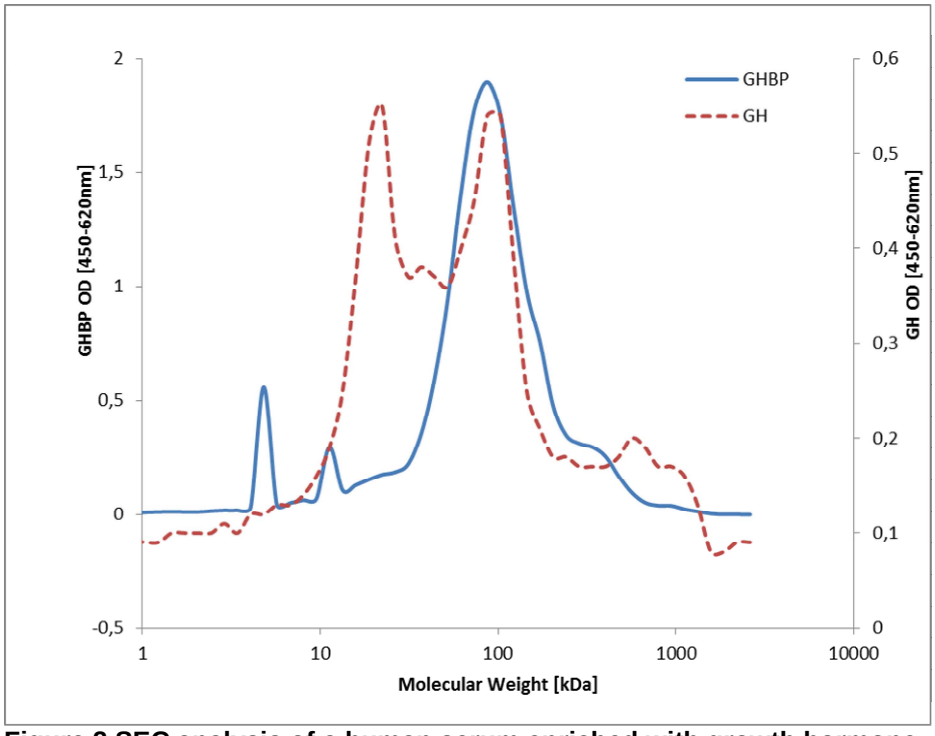

Figure 2 SEC analysis of a human serum enriched with growth hormone
A human serum was enriched with 3 µg/mL recombinant growth hormone (GH). After an incubation period of >20 h at 4°C. the enriched serum was separated by size exclusion chromatography (Superdex 10/300; Flow rate: 0.5 ml/min; Fraction size: 0.4 mL/min). GHBP and GH were measured after dilution in Mediagnost Dilution buffer by Mediagnost GHBP-Antiserum and Mediagnost GH ELISA E022.

The analysis reveals that GHBP antibodies detect a signal at 100 kDa and a small shoulder could be interpreted as an additional signal at 340 kDa. As expected GH is detected at 22 kDa (unbound GH), at 100kDa and a second peak appears >340 kDa. This shows that the protein detected by GHBP antibodies binds GH, which indicates a GH-binding property of the detected protein. The expected molecular weight of a GH/GHBP complex is 80 kDa, slightly less than the size of the GH/GHBP complex the Mediagnost antibodies detect. This is probably caused by methodological variability or differential glycosylation of native GHBP.

12.3 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 2.

Table 2: Recovery of recombinant human GHBP in serum

[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

12.4 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 0.652) an intra-assay variability of 4.38% was detected.

Inter-Assay-Variation

Serum samples where measured in independent assays. On average the coefficient of variation was 7.72% (Range 3.08 – 10.67%). Exemplary results are shown in table 3.

Table 3 : Inter-Assay-Variation

	Number of measurements	Mean value [ng/mL]	Standard Deviation [ng/mL]	VC (%)
Sample 1	7	12.61	0.39	3.08
Sample 2	8	21.21	1.8	8.47
Sample 3	8	15.66	1.02	6.5

12.5 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 (Figure 3).

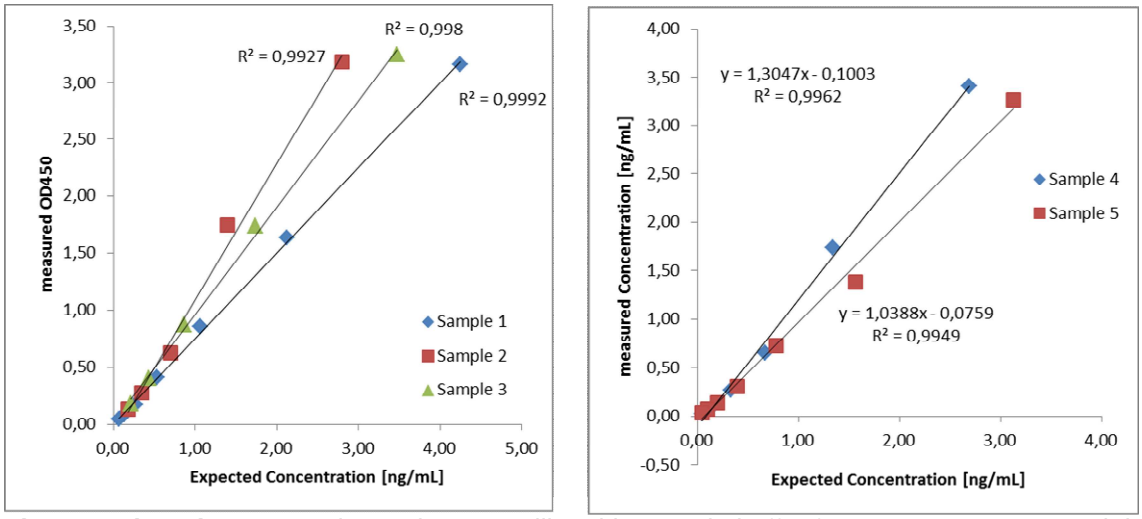

Figure 3 Linearity:. Several samples were diluted in sample buffer from 1:5 up to 1:320 and the absolut signal or the recalculated concentration are shown in comparison to the expected concentration.

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%.

International Assay Description

A-F	**STD**	**Rec in 750 µL BUF PP**	**-**
KS1	**Control**	**Rec in 250 µL BUF PP**	**1:21 DILU BUF PP**
KS2	**Control**	**Rec in 250 µL BUF PP**	**1:21 DILU BUF PP**
WP	**WASHBUF 20x**	**-**	**1:20 DILU A. dest.**
-	**SPE**		**1:21 DILU BUF PP**
-	**°C 20-25 °C**		

100 µL	**BUF PP**	A1/A2
100 µL	**STD A (0.05 ng/mL)**	B1/B2
100 µL	**STD B (0.1 ng/mL)**	C1/C2
100 µL	**STD C (0.5 ng/mL)**	D1/D2
100 µL	**STD D (1.0 ng/mL)**	E1/E2
100 µL	**STD E (1.5 ng/mL)**	F1/F2
100 µL	**STD F (4 ng/mL)**	G1/G2
100 µL	**CONTROL KS1 1:21 DILU BUF PP**	H1/H2
100 µL	**CONTROL KS2 1:21 DILU BUF PP**	A3/A4
100 µL	**SPE 1:21 DILU BUF PP**	
	TAPE	
	1 h °C 20-25 350 rpm	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**Ab AK**	
	TAPE	
	1 h °C 20-25 350 rpm	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**CONJ EK**	
	TAPE	
	0.5 h °C 20-25 350 rpm	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**SUBST TMB S**	
	0.5 h °C 20-25	
	H_2SO_4 SL	
	MEASURE	