Version 07-11/24

User's Manual

Human Insulin-like growth factor I (IGF-I) ELISA Kit

REF **DEIA-NB24-08**

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

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PRODUCT INFORMATION

Intended Use

The Human IGF-I ELISA Kit is intended to be used for the measurement of human IGF-I in serum and plasma samples. In combination with growth retardation and other clinical symptoms the results of this test system can be used as supplementary data to assess disturbances of the growth hormone axis.

General Description

Insulin-like growth factors (IGF) I and II play a pivotal role in regulating the proliferation, differentiation and specific functions of many cell types. IGF-I is identical with Somatomedin C (Sm-C) and has a molecular weight of 7649 Dalton. Its major regulators are growth hormone (GH) and nutrition, although its production in specific tissues is affected by a multitude of tropic hormones and other peptide growth factors. In contrast to many other peptide hormones, IGFs are avidly bound to specific binding proteins (IGFBP). The seven classes of IGFBPs which are known at present either bind IGF-I and IGF-II with similar affinities or show a preference for IGF-II . A major problem of IGF-I measurement results from the interference of IGFBPs in the assay. Direct determinations in untreated serum samples give false values because of the extremely slow dissociation of the IGF-I/IGFBP-3 complexes during the assay incubation.

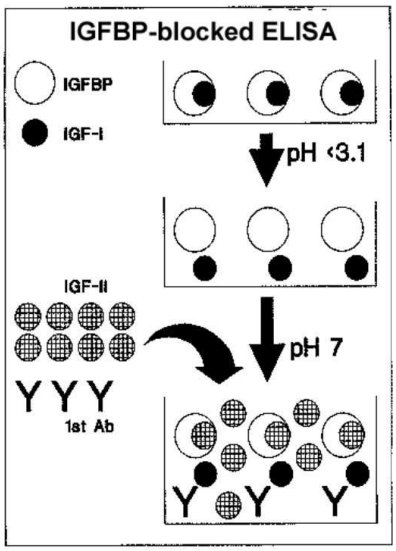
Therefore, various techniques were applied to physically separate IGF-I from its binding proteins before measurement, including (a) size exclusion chromatography under acidic conditions, (b) solid-phase extraction and (c) acid-ethanol extraction. These techniques, however, are either inconvenient or time-consuming or give incomplete and not reproducible recoveries. The most widely used method is the acid-ethanol extraction with a recovery of only 70-80 % of IGFBP-bound IGF-I as a result of co-precipitation. The absolute results of such an extraction are therefore false low. The extraction removes the IGFBPs only insufficiently and leads to reduction in sensitivity of the assay due to pre-dilution of the samples by the extraction procedure. Furthermore, the remaining IGFBP may still interfere in the assay. In addition, the acid-ethanol extraction is ineffective in specimens other than serum or plasma (e.g. cell culture media), in which determination of IGF-I is already difficult enough due to the fact that IGFBPs are frequently present at large excess. To avoid these difficulties, an uncomplicated assay was developed, in which special sample preparation is not required before measurement.

Principles of Testing

The Human IGF-I ELISA Kit is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The IGF-I in the sample binds to the immobilized first antibody on the microtiter plate, the biotinylated and Streptavidin-Peroxidase conjugated second specific anti-IGF-I-Antibody binds in turn to the immobilized IGF-I. In the closing substrate reaction, the turn of the colour will be high specific catalysed, quantitatively depending on the IGF-I-level of the samples.

In order to dissociate IGF-I from the IGFBPs, the samples must be diluted in an acidic buffer (Sample Buffer SB) (Figure below). The diluted samples are then pipetted into the wells, by this the pH-value will be neutralized. After neutralization of the samples, the excess IGF-II occupies the IGF-binding sites of the binding proteins, thus allowing the measurement of resulting free IGF-I. With this method, the IGFBPs are not removed, but their function and therefore their interference in the assay is neutralized. Due to the extremely low cross-reactivity of the IGF-I antibody with IGF-II, the excess of IGF-II does not disturb the interaction with

IGF-I. The test runs like a conventional ELISA using a Streptavidin-Peroxidase-Enzyme Conjugate.



Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with mouse-anti-hIGF-I-antibody. Wells are separately breakable. (8x12) wells
2. **Calibrators (CAL A-E):** lyophilized, (recombinant human hIGF-I), concentrations are given on vial labels and on quality certificate. 5 x 500 µL
3. **Control Serum 1 (CTR1):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 500 µL
4. **Control Serum 2 (CTR2):** lyophilized, (human serum), concentration is given on quality certificate. 1 x 500 µL
5. **Antibody Conjugate (DET):** ready for use, contains goat biotinylated anti-hIGF-I antibody. 1 x 9 mL
6. **Enzyme Conjugate (EC):** ready for use, contains HRP (Horseradish-Peroxidase)-labeled Streptavidin. 1 x 12 mL
7. **Sample Buffer (SB):** ready for use. 1 x 25 mL
8. **Washing Buffer (WB):** 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 (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. The IGF-I 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: 10 µL

4. Sample stability

In firmly closable sample vials

• Storage at 20-25°C: max. 24 hours

• Storage at -20° C: min. 2 years

• Freeze-thaw cycles max. 3

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 and 200 µg/mL or 1 mg/mL. However, the use of hemolytic, lipemic or icteric samples should be validated by the user.

6. Sample dilution

• Dilution: 1:21 with Sample Buffer SB

• Pipette 200 µL Sample Buffer SB in PE-/PP-Tube (application of a multi-stepper is recommended in larger series); add 10 µL sample (dilution 1:21). After mixing use 2 x 20 µL of this dilution in the assay.

• Attention: serum and plasma samples must be diluted at least 1:10 in Sample Buffer SB in order to achieve sufficient acidification of the samples.

• Depending on the expected IGF-I values the samples can be diluted higher in Sample Buffer SB.

• Sample stability after dilution of the sample: maximum 2 hours 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 1mL Sample Buffer SB. After resuspension, the calibrators are diluted according to a gradient - A (2 ng/mL), B (5 ng/mL), C (15 ng/mL), D (30 ng/mL), E (50 ng/mL), which are prepared for immediate use.
2. The Controls CTR1 and CTR2 are reconstituted with 500 µL Sample Buffer SB. After reconstitution dilute the Controls CTR1 and CTR2 with the Sample Buffer PP in the same ratio (1:21) as the sample. Mix directly and use within max. 120 min.

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 Conjugate DET and the Enzyme Conjugate EC 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 (1:21 diluted), Controls CTR1 and CTR2 (1:21 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 80ul Antibody Conjugate DET in all wells used. Aliquot 20ul 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 for 1 hour.
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 Enzyme Conjugate EC 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.
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 within 15 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

The International Standard for hIGF-I, WHO NIBSC Code 02/254 was used as calibrator material and the following IGF-I concentrations are used.

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 absorbance of all other samples, controls 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 calibrator 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 IGF-I concentration in ng/mL of the samples can be calculated by multiplication with the respective dilution factor.

Exemplary calculation of IGF-I concentrations

Sample dilution: 1:21

Measured extinction of your sample 0.2695

Measured extinction of the blank 0.0165

Your measurement program will calculate the IGF-I 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 IGF-I concentration in the sample:

$0.253 = -0.0004x^2 + 0.0727x - 0.0471$

$4.57 = x$

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

$4.57 \text{ ng/mL} \times 21 = 96 \text{ ng/mL}$

Interpretation Of Results

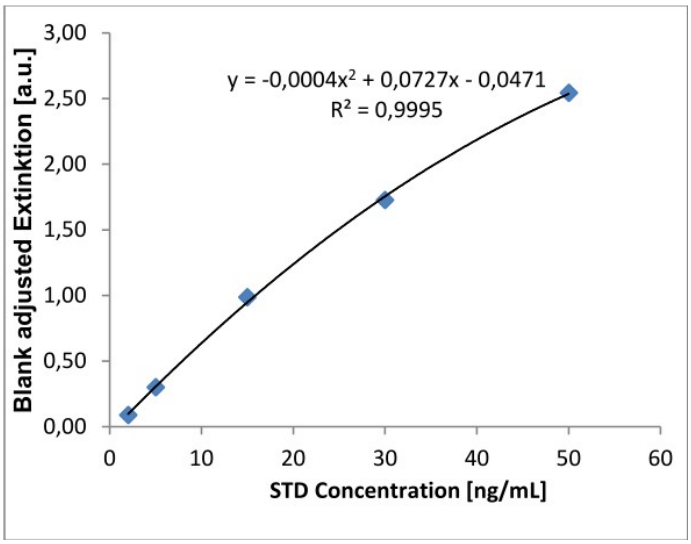
The test results should not be the only base for therapeutic decisions. The results should be interpreted in regard to anamnesis, further clinical observations and results of other diagnostic investigations. Further, it is recommended to establish reference and cut-off values corresponding to the relevant group of patients for each laboratory. Please consider the international and national guidelines for diagnosis and treatment of growth hormone deficiency / acromegaly.

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	2	10	30	70	100
$OD_{(450\text{-}620\ nm)}$	0.00	0.088	0.299	0.985	1.727	2.543

The exemplary shown standard 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

Three samples have been measured severalfold in the same assay. The measured coefficient of variation (CV) is 5.81% on average.

	Determinations [n]	Mean value [ng/mL]	SD	CV [%]
Sample 1	18	144.8	9.63	6.65
Sample 2	18	140.79	7.15	5.08
Sample 3	18	138.02	7.86	5.69

Inter-Assay and Lot-to-Lot Variance

Serum samples where measured in independent assays. Further, five samples were also tested repeatedly four years in eight different lots. The variability on average is 8.57% (6.8 – 10.5%).

	Determinations [n]	Mean value [ng/mL]	SD	CV [%]
Sample 1	8	81	5.34	6.56
Sample 2	16	192	12.38	6.43
Sample 3	17	498	27.52	5.53

Sensitivity

Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the blank + 2SD. The analytical sensitivity of the Human IGF-I ELISA Kit is 0.091 ng/mL as mean, in 19 independent determinations values from 0.03 ng/mL to 0.2 ng/mL were found.

Specificity

The measurements of Human IGF-I ELISA Kit cross reactivity with IGF-II, Insulin and C-Peptide. These IGF-related proteins were added to assay buffer in the indicated concentration and the solution was applied as sample without any further dilution. The concentration measured within the blank without any protein was 0.78 µg/L. Thus, neither IGF-II nor Insulin or C-Peptide are measured by the Human IGF-I ELISA Kit (see table below).

Added C-Peptide [µg/L]	Measured IGF-I [µg/L]	Added Insulin [µg/L]	Measured IGF-I [µg/L]	Added IGF-II [µg/L]	Measured IGF-I [µg/L]
500	0.73	100	0.78	1250	0.77
100	0.78	10	0.77	750	0.73
10	0.77	1	0.76	250	0.77
0	0.78	0	0.78	0	0.78

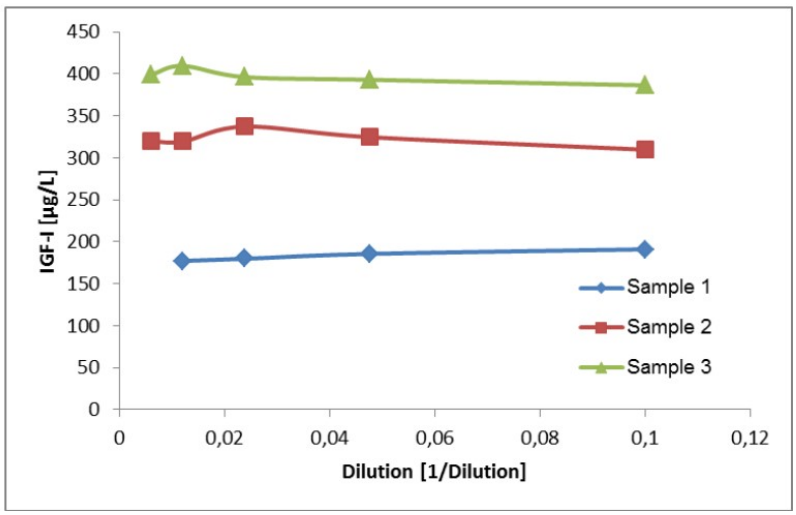
Cross reactions with animal samples

Several commercially available animal sera have been used as samples in this assay and therewith it is proven, that the test can be used as heterologous assay for IGF-I measurement in serum samples of primates, cattle, pig, sheep, horse, donkey, goat, dog, cat, rabbit and guinea pig. For the determination of IGF-I in chicken, rat and mouse sera is this kit not usable.

Species specific calibration has to be done by the user. To determine exact species-specific IGF-I concentrations, the test system must be calibrated respectively: for example, using bovine IGF-I in a known concentration.

Linearity

Linearity was tested by dilution of native sera with different IGF-I contents (Sample 1-3). The amount of measured IGF-I was recalculated and is shown in Figure below.



Recovery

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

IGF-I [µg/L]	Sample 1	Sample 2	Sample 3	Sample 4
Sample	138	172	133	180
+ IGF-I 200	287	372	-	-
+ IGF-I 400	-	-	539	591
% Recovery	85	100	101	102

Interferences

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

	Triglyceride (100 mg/mL)	Bilirubin (100 µg/mL)	Hemoglobin (1 mg/mL)
Serum 1	93	90	97
Serum 2	100	101	110
Serum 3	120	120	104

Influence of binding proteins on IGF-I measurement was exemplarily elucidated with IGFBP-3. Different amounts of IGF-I and 3 or 6 mg/L of IGFBP-3 were added to sample buffer (pH 2) and phosphate based saline buffer (pH 7.4). After a short incubation of 15 minutes at room temperature these samples were diluted and applied to the Human IGF-I ELISA Kit as described in the package insert. In case of sample buffer IGFBP-3 up to 6 mg/L did not interfere with IGF-I measurement. But without acidification of the sample a strong interference of IGFBP-3 with IGF-I measurement was detected.

IGFBP-3	Sample Buffer		
	50 µg/L IGF-I	100 µg/L IGF-I	300 µg/L IGF-I
-	46.38	116.14	358.1
3 mg/L	47.33	115.83	384.15
6 mg/L	52.32	123.38	355.41
IGFBP-3	**Phosphate buffered Saline**		
	50 µg/L IGF-I	**100 µg/L IGF-I**	**300 µg/L IGF-I**
-	34.2	90.23	349.04
3 mg/L	7.4	12.16	152.14
6 mg/L	7.4	10.12	48.15

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 1, Control Serum 2.

   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

IGF-I levels depend to a great degree on GH secretion. Diminished IGF-I values, however, do not prove GH deficiency, because a number of other factors can influence the plasma concentration of IGF-I and must therefore be taken into account in order to make a correct interpretation. IGF-I levels decrease during fasting (more than 1 day), as a result of malnutrition, malabsorption, cachexia, impaired hepatic function, or in hypothyroidism and untreated diabetes mellitus. They may also be low in chronic inflammatory disease and malignancies. IGF-I levels are high in states of accelerated sexual development. In clinical situations with hyperprolactinemia or in patients with craniopharyngioma, normal levels may be observed despite GH deficiency. In late pregnancy, IGFI levels are moderately elevated.

The CD Human IGF-I ELISA Kit is based on mono- and polyclonal antibodies. Generally the result of any immunological test system can be influenced by heterophilic antibodies, anti-species antibodies or rheumatic factors. The assay design reduces these potential influences to a minimum but they cannot be excluded in any case.