Instructions for Use

IGFBP-3 ELISA

Enzyme Immunoassay for the quantitative determination of Insulin-like Growth Factor Binding Protein 3 (IGFBP-3) in human serum and plasma.

REF **MD58031**

Σ **96**

2-8°C

EU: IVD CE

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IGFBP-3 ELISA	96 Determinations
Principle of the test	Sandwich ELISA
Duration (incubation period)	2.5 h
Antibody-HRP-Conjugate	ready for use
Buffer and Substrate	ready for use
Standards	5 single standards: 0.4 - 30 ng/mL, lyophilized, human IGFBP-3
Assay Range	0.03 – 15150 ng/mL
Control	2 control sera, lyophilised
Sample	human serum / plasma
Required sample volume	10 µL
Sample dilution	1:505
Analytical sensitivity	0.03 ng/mL
average Intra- / Inter-Assay Variance	1.9% / 5.7%
Reference Values	Blum W.F et.al.1990 Insulin-Like Growth Factors and Their Binding Proteins. In: Ranke MB, Mullins P.E.(ed): Diagnostics of Endocrine Function in Children and Adolescents. Basel, Karger, 2011, pp.157-181

1 INTENDED USE

This enzyme immunoassay kit is suited for measuring IGFBP-3 in human serum, EDTA and Heparin-plasma for diagnostic and scientific purposes.

2 INTRODUCTION

Insulin-like growth factors (IGF)-I and -II are bound to specific binding proteins (IGFBPs) in the circulation. To date, at least six binding proteins can be distinguished on the basis of their amino acid sequence. They are designated as IGFBP-1, IGFBP-2, ... IGPBP-6 (1). The predominating IGFBP in blood is IGFBP-3, which largely determines the total IGF-I and IGF-II concentration. In contrast to the other binding proteins, IGFBP-3 has the property to associate with an acid-labile subunit (ALS) after binding of either IGF-I or IGF-II (3-5). Most of the IGFBP-3 in plasma is present as high molecular weight ternary complex, however, small amounts of free IGFBP-3 are also found (6,7).

The development of a specific immunoassays for IGFBP-3, which detects IGFBP-3 in the ternary complex, provided new in-sights into IGFP-3 regulation (6-9). On the basis of these findings serum IGFBP-3 has been proven to be an additional useful test in the repertoire of diagnostic tools for evaluation of growth disorders (7,8).

Several factors besides GH influence IGFBP-3 levels: age including sexual development, nutrition, hypothyroidism, diabetes mellitus, liver function and kidney function. IGFBP-3 levels are decreased by malnutrition, although less than IGF-I, in hypothyroidism, in diabetes mellitus and in hepatic failure (6-8), but are increased in chronic renal failure (6,10,11). Measurement over 24 hours revealed no circadian rhythm (12,13). For clinical practice, the most important regulatory factor is GH. Single IGFBP-3 measurements correlate significantly with the logarithm of the integrated spontaneous GH secretion (8,14). In patients with GH deficiency, IGFBP-3 levels are subnormal and increase gradually to within the normal range after several days of GH administration (7,8). The slow response to GH and constant circadian levels during chronic daily application of GH (13) suggest that IGFBP-3 reflects the GH secretory state over days.

The major advantages of IGFBP-3 over IGF-I are:

1. No extraction step is required prior to measurement thus improving test accuracy by simplifying the assay procedure.
2. The normal range in young children is comparatively high making the detection of subnormal levels more reliable.
3. Patients with GH deficiency have subnormal IGFBP-3 levels. In contrast, most of the small statured children with normal GH secretion have levels within the normal range (Figure 1). The separation of these two groups is easy. In small statured children IGFBP-3 levels rise to normal range within several days of GH administration and remain normal during continuous GH treatment (Figure 2). Therefore, serum IGFBP-3 measurements are also suited for evaluating the potential of a patient to respond to GH and for GH therapy monitoring (19). In other patients of severe short stature, e.g. Ullrich-Turner syndrome or Silver-Russell syndrome, IGFBP-3 levels were found normal (8) reflecting normal GH secretion.

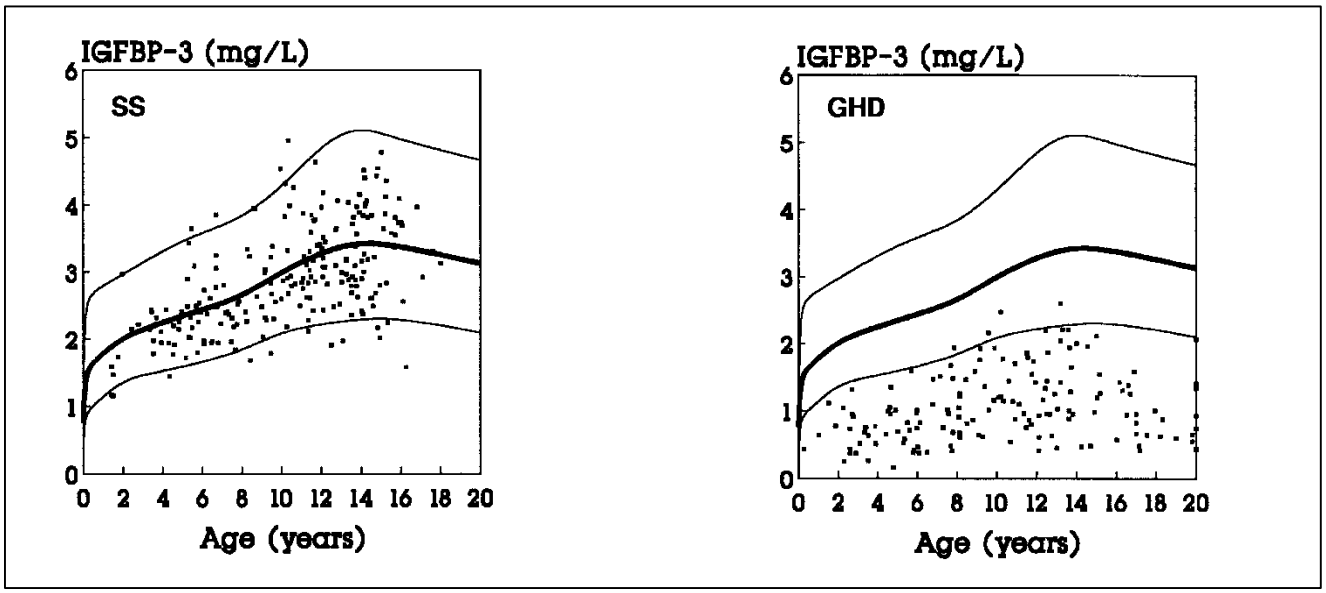

Fig. 1: Serum IGFBP-3 levels in patients with short stature without GH deficiency (SS: constitutional delay of growth and adolescence, familial short stature, intra-uterine growth retardation) and in idiopathic or organic GH deficiency (GHD). The normal range is given by the 5th, 50th and 95th percentile.

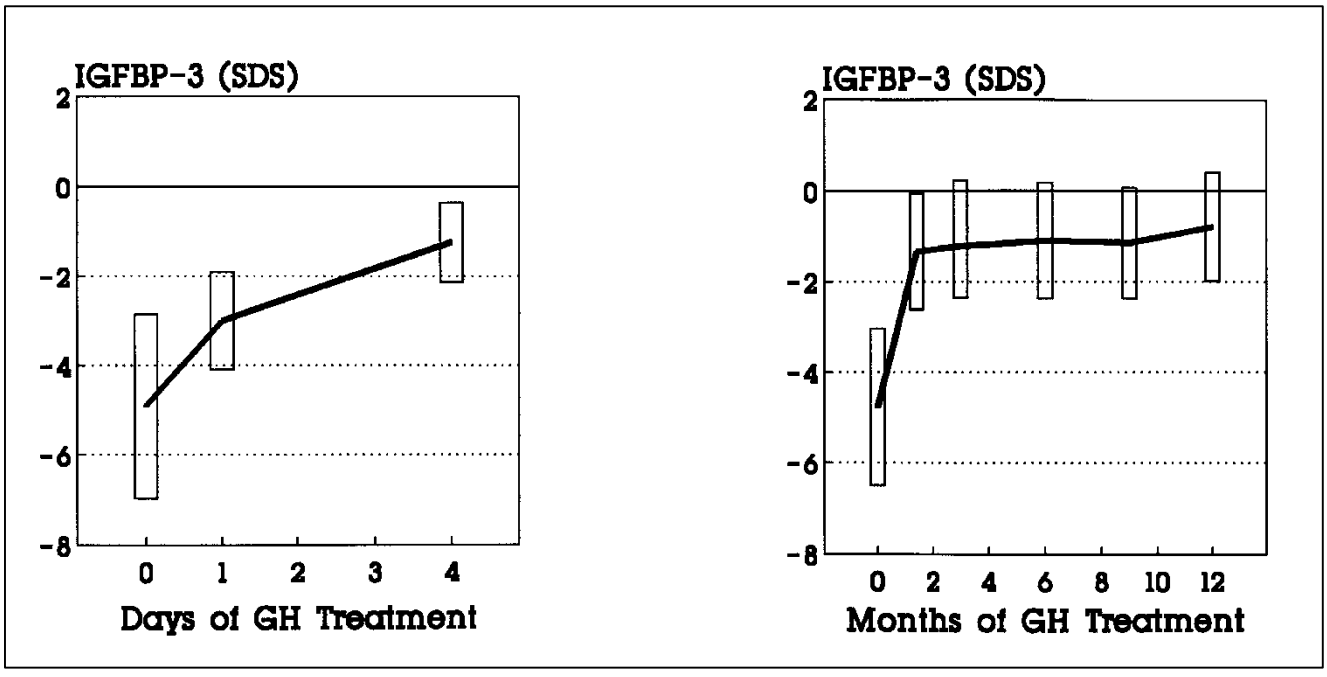

Fig. 2: IGFBP-3 levels in GH deficient children before and during GH treatment. Because of the age-dependence, values are given as the mean of standard deviation scores (SDS).

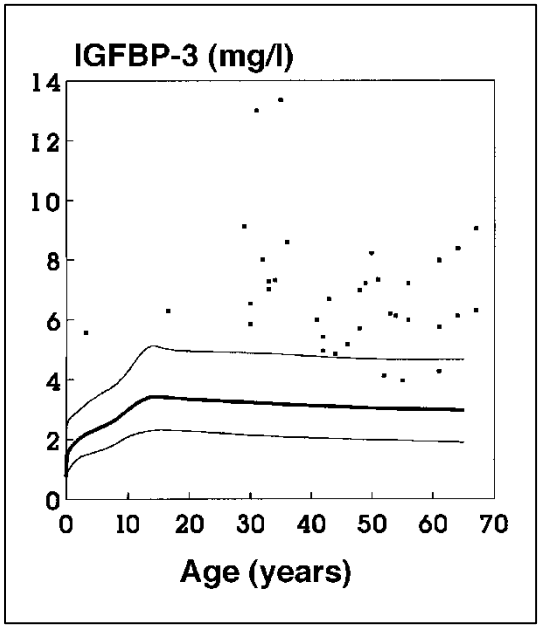

Fig. 3: Serum IGFBP-3 levels in acromegaly. The normal range is given by the 5th, 50th and 95th percentile.

In normal tall children and adolescents without excessive GH secretion or in patients with Sotos syndrome, IGFBP-3 levels are normal or slightly increased. In contrast, children with pituitary gigantism or adults with acromegaly have clearly elevated levels (Figure 3) (6,15) that normalize on successful treatment. Therefore, IGFBP-3 is also a useful parameter for the detection of excessive GH secretion and monitoring therapy efficacy. In precocious puberty, IGFBP-3 levels are clearly increased by chronological age, whereas patients with premature thelarche have IGFBP-3 levels in the upper normal range (15).

3 ASSAY PRINCIPLE

The IGFBP-3 ELISA is a so-called Sandwich-Assay. It utilizes two specific antibodies of high affinity. First the IGFBP-3 in the sample binds to the immobilized antibody on the microtiter plate. In the following step, the complex of biotinylated anti-IGFBP- 3-Antibody and Streptavidin-Peroxidase binds in turn to the immobilised IGFBP-3. 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.

4 WARNINGS AND PRECAUTIONS

For In Vitro Diagnostic Use only. For Professional use only.

The ELISA is suitable only for in vitro diagnostics 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. IBL 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 1-2 CAL A-E

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 ANTIBODY, SAMPLEBUF, WASHBUF

Contain as preservative **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 TMB SUBS
The TMB-Substrate 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/ vapours/ 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 TMB STOP
The Stopping solution contains 0.2 M acid sulphur 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.

5.2 Specimen collection

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

5.3 **Required** sample volume: **10 µL**

5.4 **Sample** stability

In firmly closable sample vials

- Storage at 20-25°C: 3 days
- Storage at -20° C: min. 2 years
- Freeze-thaw cycles max. 10

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. 10 Freezing-Thawing showed no effect on samples.

5.5 Interference

Triglyceride, bilirubin and hemoglobin in the sample do not interfere to a concentration of 100 mg/mL, 100 µg/mL or 5 mg/mL, respectively. However, the use of haemolytic, lipemic or icteric samples should be validated by the user.

5.6 Sample dilution

- Dilution: **1:505** with Sample Buffer SAMPLEBUF
- Pipette **1 ml Sample Buffer** SAMPLEBUF (red colored) in PE-/PP-Tubes (application of a multi- stepper is recommended in larger series), add **10 µL Serum**- or **Plasma** (dilution factor 101). Add **400 µL** Sample Buffer SAMPLEBUF in another PE-/PP-tube and **100 µL** of the thoroughly mixed first dilution (dilution factor 5). After mixing use **50 µL** of this 1:505 diluted solution **within 1 hour per determination** in the assay.
- Sample stability after dilution of the sample: maximum 1 hour at 20-25°C.

6 MATERIALS

6.1 Materials provided

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

1	MTP	**(8x12) wells**	**Microtiter plate,** ready for use, coated with rabbit-anti-hIGFBP-3- antibody. Wells are separately breakable.
2	CAL A LYO CAL B LYO CAL C LYO CAL D LYO CAL E LYO	**5 x 1 mL**	**Standards**, lyophilized, (human IGFBP-3), concentrations are given on vial labels and on the QC-certificate.
3	CONTROL 1 LYO	**1x 250 µL**	**Control Serum 1,** lyophilised, (human serum), concentration is given on the QC-certificate.
4	CONTROL 2 LYO	**1x 250 µL**	**Control Serum 2,** lyophilised, (human serum), concentration is given on the QC-certificate.
5	ANTIBODY	**1 x 12 mL**	**Antibody-HRP-Conjugate,** ready for use, contains rabbit biotinylated anti-hIGFBP-3 antibody**.**
6	SAMPLEBUF	**1 x 120 mL**	**Sample Buffer,** red color, ready for use, **Please shake before use!**
7	DILBUF	**1 x 30 mL**	**Dilution Buffer,** ready for use, **Please shake before use!**
8	WASHBUF CONC	**1 x 50 mL**	**Washing Buffer,** 20-fold concentrated solution
9	TMB SUBS	**1 x 12 mL**	**Substrate,** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised H_2O_2 Tetramethylbencidine.
10	TMB STOP	**1 x 12 mL**	**Stopping Solution,** ready for use, 0.2 M sulphuric acid.
11		**2 x**	**Sealing Tape,** for covering the **microtiter plate.**

6.2 Materials required, but not provided

- Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer **(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-E** and Control Sera **1** and **2** 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 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 CAL A – E LYO and Controls CONTROL 1 LYO and CONTROL 2 LYO are reconstituted with the Sample Buffer SAMPLEBUF. 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 1 and 2** with the Sample Buffer SAMPLEBUF in the same ratio (1:505) as the sample. The required volume of Washing Buffer is prepared by 1:20 dilution of the provided 20fold WASHBUF CONC concentrate with Aqua dest.

Assay Procedure

When performing the assay, Blank, Standards **A-E**, Control Serum **1 and 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-HRP-Conjugate as well as the succeeding Substrate Solution should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution should be added to the plate in the same order as Substrate Solution
All determinations (Blank, Standards **A-E**, Control Sera **1 and 2** 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 TMB SUBS, stabilised H_2O_2- 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 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 ASSAY PROCEDURE

Preparation of reagents	Reconstitution:	Dilution
A-E Standards	in **1 mL** SAMPLEBUF	-
Control Serum 1 CONTROL 1 LYO	in **250 µL** SAMPLEBUF	**1:505** with SAMPLEBUF
Control Serum 2 CONTROL 2 LYO	in **250 µL** SAMPLEBUF	**1:505** with SAMPLEBUF
Washing Buffer WASHBUF	-	**1:20** with **Aqua dest.**
Sample dilution: with Sample Buffer SAMPLEBUF **1:505**		
Before assay procedure bring all reagents to room temperature **20-25°C**.		
Assay Procedure in Double Determination:		

Pipette	Reagents	Position
50 µL	Dilution Buffer DILBUF	Pipette **in all required number of wells**
50 µL	Sample Buffer SAMPLEBUF as Blank	A1/A2
50 µL	Standard **A** (**0.4 ng/mL**)	B1/B2
50 µL	Standard **B** (**2 ng/mL**)	C1/C2
50 µL	Standard **C** (**6 ng/mL**)	D1/D2
50 µL	Standard **D** (**15 ng/mL**)	E1/E2
50 µL	Standard **E** (**30 ng/mL**)	F1/F2
50 µL	Control Serum **1** (1:505 diluted)	G1/G2
50 µL	Control Serum **2** (1:505 diluted)	H1/G2
50 µL	Sample (1:505 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 **diluted Washing Buffer / well**	In each well
100 µL	Antibody-POD-Conjugate ANTIBODY	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 diluted Washing Buffer **/ well**	In each well
100 µL	Substrate Solution TMB SUBS	In each well
Incubation: 30 Minutes in the Dark at 20-25°C		
100 µL	Stopping Solution TMB STOP	In each well
Measure the absorbance within 30 min at **450 nm** with ≥ 590 nm as reference wavelength.		

9 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 standards 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.

9.1 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.

10 EVALUATION OF RESULTS

10.1 Establishing of the standard curve

The standards provided contain the following concentrations of hIGFBP-3

Standard	A	B	C	D	E
ng/mL	0.4	2	6	15	30

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 IGFBP-3 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.**

10.2 Example of a 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	0.4	2	6	15	30
$OD_{(450\text{-}620\ nm)}$	0.204	0.254	0.453	0.911	1.706	2.390

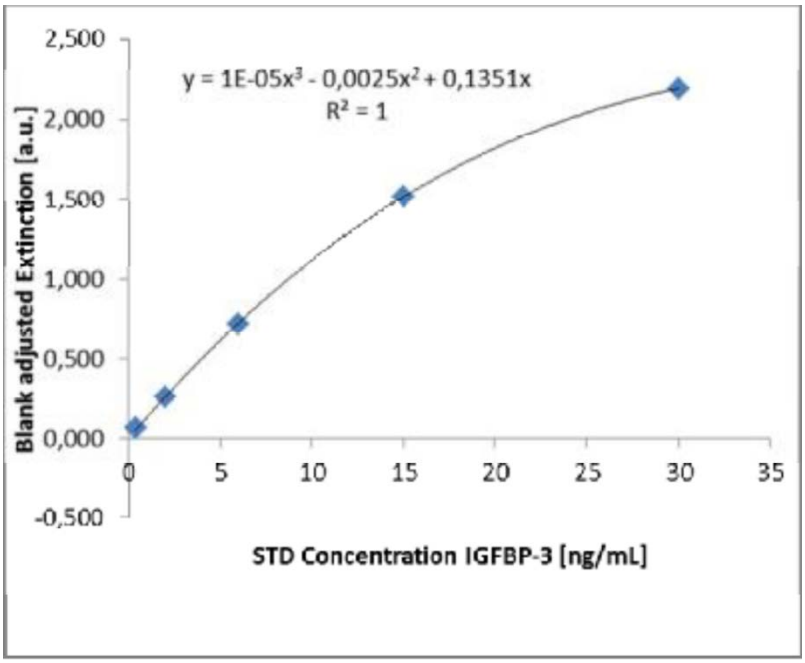

Fig. 4: Exemplary standard curve

The exemplary shown standard curve in Figure 4 **cannot** be used for calculation of your test results. You have to establish a standard curve for each test you conduct!

10.3 Exemplary calculation of IGFBP-3 concentrations

Sample dilution: 1:505

Measured extinction of your sample 0.975
Measured extinction of the blank 0.204

Your measurement program will calculate the IGFBP-3 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 (here: polynomial x^{rd} degree).

In this exemplary case the following equation is solved by the program to calculate the IGFBP-3 concentration in the sample:

$0.771 = 1E\text{-}05x^3 – 0.0025x^2 + 0.1351x$
$6.617 = x$

If the dilution factor (**1:505**) is taken into account the IGFBP-3 concentration of the undiluted sample is
6.617 ng/mL × 505 = 3342 ng/mL = 3,342 mg/L

10.4 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.

11 LIMITATION OF PROCEDURE

IGFBP-3 levels are strongly dependent on GH secretion. However, a number of factors influence its plasma concentration and should be taken into account for appropriate interpretation. Plasma levels decrease during fasting (more than 1 day), in malnutrition, malabsorption, cachexia, impaired hepatic function, hypothyroidism, and diabetes mellitus. They may also be decreased in chronic inflammatory disease and malignancy. Levels are increased in states of impaired renal function and precocious puberty. In clinical situations with hyperprolactinemia or in patients with craniopharyngeoma, normal levels may be observed despite GH deficiency.
In certain physiological (e.g. pregnancy) and pathological states, IGFBP-3 may be degraded to smaller molecular size compounds (16,17) by specific proteases which affect IGFBP patterns seen in Western ligand blotting, but in general only have little influence on the outcome of ELISA determinations.
The IGFBP-3 ELISA is based on polyclonal rabbit antibodies. Generally, this technique is sensible to heterophilic antibodies in the sample. The influence of heterophilic antibodies is reduced by assay design, but cannot be excluded completely.

12 REFERENCE VALUES

IGFBP-3-levels are strongly age-dependent in children, less so in adults. The normal ranges in various age-groups which were log-normally distributed are given in table 1 by the percentiles (see Appendix). A graphic presentation is shown in Figure 5 and 6. It is recommended for each laboratory to establish its own normal range.

Tab. 1: Serum levels of IGFBP-3 in healthy subjects at various ages. Individuals between 7 and 17 years of age were classified according to gender, as the pubertal peak occurs almost 2 years earlier in girls than in boys.

	Percentiles													
Age group	**0.1**	1	**5**	10	**20**	30	**40**	50	**60**	70	**80**	90	**95**	99
0-1 week	**0.25**	0.33	**0.42**	0.48	**0.57**	0.64	**0.70**	0.77	**0.85**	0.93	**1.05**	1.23	**1.41**	1.81
1-4 weeks	**0.49**	0.62	**0.77**	0.86	**0.99**	1.10	**1.19**	1.29	**1.40**	1.52	**1.68**	1.93	**2.16**	2.68
1-3 months	**0.55**	0.70	**0.87**	0.98	**1.13**	1.25	**1.36**	1.48	**1.61**	1.75	**1.94**	2.23	**2.52**	3.14
3-6 months	**0.64**	0.80	**0.98**	1.10	**1.25**	1.38	**1.49**	1.61	**1.74**	1.88	**2.07**	2.37	**2.65**	3.24
6-12 months	**0.71**	0.88	**1.07**	1.19	**1.35**	1.48	**1.60**	1.72	**1.85**	2.00	**2.19**	2.49	**2.76**	3.36
1-3 years	**1.02**	1.21	**1.41**	1.53	**1.69**	1.82	**1.94**	2.05	**2.17**	2.31	**2.48**	2.74	**2.98**	3.47
3-5 years	**1.08**	1.30	**1.52**	1.66	**1.84**	1.99	**2.12**	2.25	**2.39**	2.55	**2.75**	3.05	**3.33**	3.91
5-7 years	**1.19**	1.42	**1.66**	1.81	**2.01**	2.16	**2.30**	2.44	**2.59**	2.76	**2.97**	3.29	**3.59**	4.2
7-9 y. boys	**1.25**	1.48	**1.73**	1.88	**2.07**	2.22	**2.36**	2.50	**2.65**	2.81	**3.02**	3.33	**3.61**	4.22
girls	**1.36**	1.61	**1.88**	2.04	**2.25**	2.42	**2.57**	2.72	**2.88**	3.06	**3.28**	3.62	**3.94**	4.58
9-11 y. boys	**1.47**	1.73	**1.99**	2.15	**2.36**	2.52	**2.66**	2.81	**2.96**	3.14	**3.35**	3.67	**3.97**	4.57
girls	**1.56**	1.90	**2.20**	2.38	**2.62**	2.80	**2.96**	3.13	**3.30**	3.50	**3.75**	4.11	**4.45**	5.16
11-13 y. boys	**1.58**	1.88	**2.19**	2.38	**2.63**	2.82	**3.00**	3.18	**3.37**	3.58	**3.84**	4.25	**4.62**	5.39
girls	**1.62**	1.90	**2.24**	2.46	**2.74**	2.97	**3.17**	3.38	**3.60**	3.85	**4.17**	4.65	**5.10**	6.02
13-15 y. boys	**1.62**	1.89	**2.24**	2.46	**2.76**	2.99	**3.20**	3.42	**3.65**	3.91	**4.24**	4.75	**5.22**	6.20
girls	**1.69**	2.03	**2.39**	2.61	**2.91**	3.14	**3.35**	3.56	**3.79**	4.04	**4.36**	4.85	**5.30**	6.24
15-17 y. boys	**1.70**	2.02	**2.36**	2.57	**2.84**	3.05	**3.25**	3.44	**3.65**	3.88	**4.17**	4.61	**5.01**	5.86
girls	**1.62**	1.93	**2.26**	2.46	**2.73**	2.93	**3.12**	3.31	**3.51**	3.74	**4.02**	4.45	**4.85**	5.67
17-20 y.	**1.58**	1.90	**2.24**	2.45	**2.72**	2.94	**3.13**	3.33	**3.54**	3.78	**4.07**	4.53	**4.95**	5.83
20-30 y.	**1.55**	1.86	**2.20**	2.41	**2.68**	2.90	**3.09**	3.29	**3.50**	3.74	**4.04**	4.50	**4.92**	5.80
30-40 y.	**1.44**	1.75	**2.08**	2.29	**2.56**	2.78	**2.98**	3.18	**3.39**	3.64	**3.95**	4.42	**4.86**	5.78
40-50 y.	**1.38**	1.68	**2.01**	2.21	**2.48**	2.69	**2.88**	3.08	**3.29**	3.53	**3.83**	4.29	**4.72**	5.63
50-60 y.	**1.34**	1.64	**1.96**	2.16	**2.42**	2.63	**2.83**	3.02	**3.23**	3.46	**3.76**	4.22	**4.65**	5.55
60-70 y.	**1.28**	1.58	**1.90**	2.10	**2.37**	2.58	**2.78**	2.98	**3.19**	3.44	**3.75**	4.23	**4.67**	5.62
70-80 y	**1.20**	1.50	**1.81**	2.00	**2.27**	2.47	**2.67**	2.87	**3.08**	3.32	**3.62**	4.09	**4.52**	5.44
> 80 y	**1.13**	1.43	**1.73**	1.92	**2.19**	2.39	**2.59**	2.79	**3.00**	3.23	**3.54**	4.00	**4.44**	5.36

Serum levels are given as mg/L y. = years

Determined with IGFBP-3 RIA (Blum et al. 1990) The values above 70 years are extrapolated.

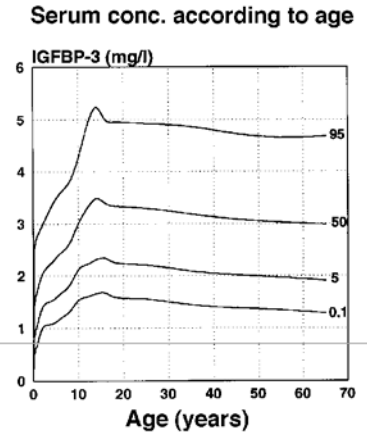

Fig. 5: Age-dependant normal values of IGFBP-3 (presented as 0.1., 5., 50., and 95. percentile)

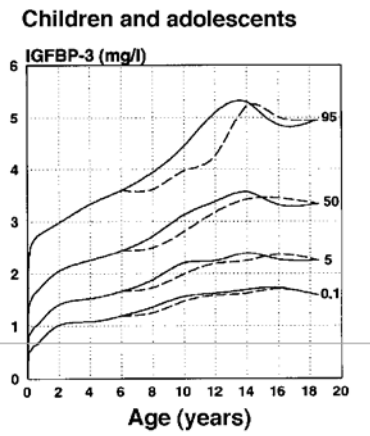

Fig. 6: Normal values of children and adolescents (girls —— boys - - - -)

13 PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

Sensitivity was assessed by measuring the blank and calculating the theoretical concentration of the 2fold standard deviation of the blank. The analytical sensitivity of the IGFBP-3 ELISA is 0.03 ng/mL.

According ICH Q2 R1 (CPMP/ICH/381/95) the limit of quantification (LoQ) is reflected by the recalculated IGFBP-3 concentration of the 10fold standard deviation of the blank, which therewith is 0.15 ng/mL.

13.2 Specificity

The cross-reactivity of the antibodies used for IGFBP-3 ELISA to homologous proteins was evaluated by diluting IGFBP-1, -2,-4,-5 and -6 in assay buffer to a concentration of 200 ng/mL and subsequent measurement of IGFBP-3. The relative cross-reactivities were ≤ 0.125%.

13.3 Reproducibility and Precision

Intra-Assay-Variation

One sample has been measured 10 times in the same assay. The results are shown in table 2. The measured coefficient of variation (CV) is on average 1.9%

Tab. 2: Intra-Assay-Variation. Three exemplary serum samples were diluted and measured 10 times within one assay.

	Sample 1	Sample 2	Sample 3
Mean [ng/mL]	3630	3789	3016
SD [ng/mL]	70.83	83.75	46.71
CV [%]	1.95	2.21	1.55
n	10	10	10

Inter-assay-Variation

Serum samples were measured in independent assays on different days. On average the coefficient of variation was 5.7%. Results are shown in detail in table 3.

Tab. 3: Inter-Assay-Variation. Serum samples were diluted as recommended (1:505) and IGFBF-3 concentration was measured in different independent assays.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
Mean [ng/mL]	2886	3525	3229	3219	4025	3293	3889	4328
SD [ng/mL]	193	178	140	237	171	177	199	322
CV [%]	6.68	5.05	4.34	7.36	4.25	5.38	5.12	7.44
n	4	10	9	7	10	10	7	10

13.4 Linearity

Linearity was proven by dilution of three different serum samples with known IGFBP-3 concentration. The IGFBP-3 concentration of the diluted sample was measured and compared with the concentration expected. Results of linear regression analysis are shown in Figure 7. None of IGFBP-3 concentrations of the dilutions (1:125 to 1:2000) deviated more than 20% of the expected value (≤ -17%).

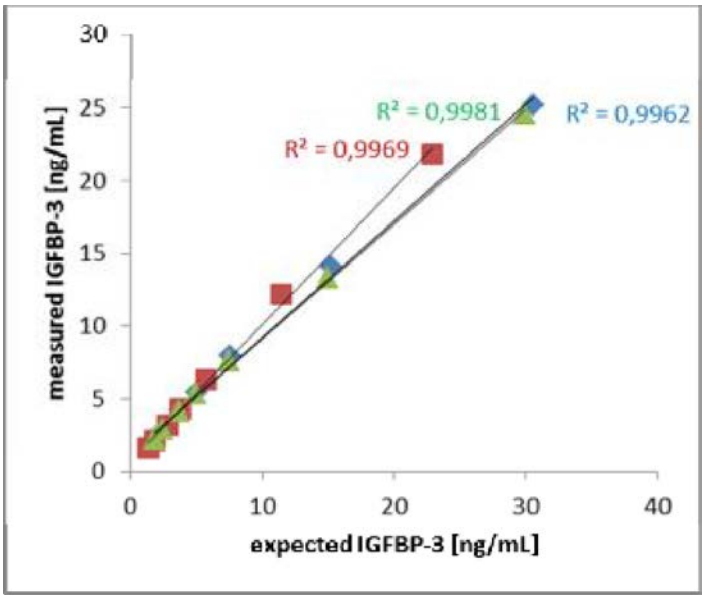

Fig. 7: Linearity. Shown are the measured concentrations in different dilutions of three serum samples.

13.5 Recovery

Serum and plasma samples were enriched with recombinant IGFBP-3 and the recovery was calculated in comparison to buffer enriched with the same amount of IGFBP-3. The native samples used had an IGFBP-3 concentration of 2684 to 3667 ng/mL and the relative recovery was 109 – 118%. Results are shown in Table 4.

Tab. 4: Recovery [%] of recombinant IGFBP-3 in native serum/plasma samples in comparison to recombinant IGFBP-3 in buffer.

IGFBP-3		Sample [ng/mL]	Sample enriched [ng/mL]	Target value [ng/mL]	Recovery [%]
Sample 1	Plasma	3641	5107	4324	118
Sample 2	Plasma	3667	4778	4350	110
Sample 3	Serum	2869	3778	3552	106
Sample 4	Serum	2684	3677	3367	109

13.6 Interference

Interference of physiological appearing substance with the IGFBP-3 measurement was investigated. Serum samples have been enriched with different concentrations of possibly interfering substances and the amount of IGFBP-3 was measured and compared with the IGFBP-3 concentration in the same sample without any enrichment. In Table 5 the relative results are shown. None of the tested substances interfered significantly with IGFBP-3 measurement.

Tab. 5: Recovery [%] in comparison to the native serum.

	Triglyceride 100 mg/mL	Bilirubin 100 µg/mL	Hemoglobin 5 mg/mL
Sample 1	89	93	81
Sample 2	87	91	106
Sample 3	88	96	93

13.7 Comparison with other assays

The IGFBP-3 ELISA was compared with the RIA test system. In total 150 different samples with IGFBP-3 concentrations of 10 – 5118 ng/mL were used. The results are shown graphically in Figure 8. The relative deviation of RIA is on average 9.00% (0.18-28.29%).

a) b)

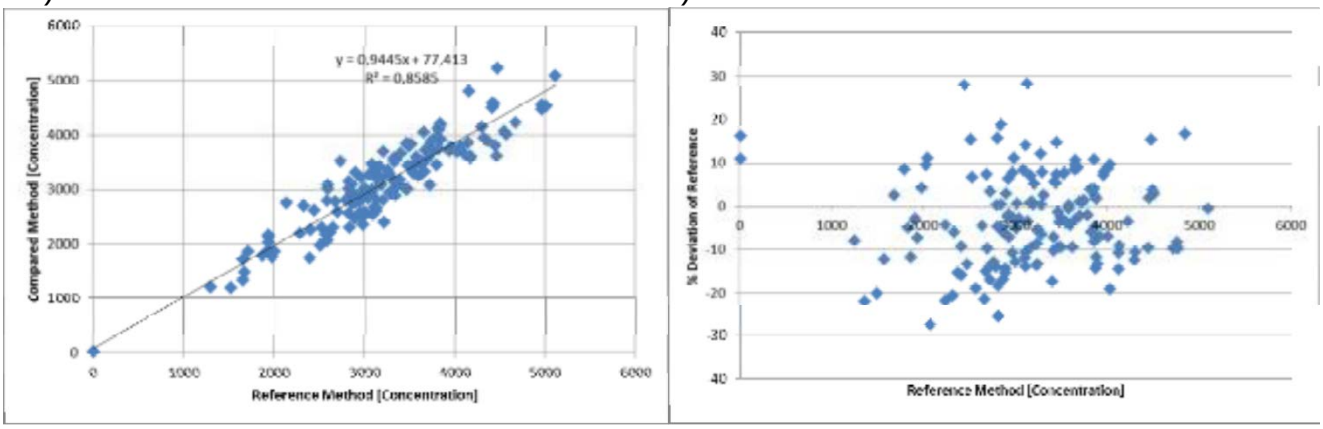

Fig. 8: Comparison of RIA to IGFBP-3 ELISA with Fab Detection Antibody. IGFBP-3 concentration was measured in 150 samples with both assays. Here results are analysed by linear regression analysis (a) and Bland-Altman plot (b).

Further, data resulting from the external quality assessment provided by DGKL/ RfB (Germany) are shown (Table 6). Here the deviation of the concentrations measured by IGFBP-3 ELISA from the method-specific and method-independent target value is shown. The deviation of the Method-specific target value is -17% and the deviation of the method-independent target value is -19% on average, which is within the acceptance criteria of the DGKL/RfB (≤ 30%).

Tab. 6: External Quality Assessment Scheme:
DGKL/RfB Germany retrospective analysis of 2013/2014 samples.

DGKL EQA		Mean	HP1/14	HP4/13	HP3/13	HP2/13	HP1/13
Measured Value [ng/mL]	Probe A	-	3608	3778	3163	3222	3144
Target Value [ng/mL]	Probe A	-	3377	3723	3532	3740	3889
Total Mean[ng/mL]	Probe A	-	3948	3899	3825	3826	3922
Δ A Targetvalue [%]	Probe A	-15.76	6.83	1.47	-10.46	-13.85	-19.16
Δ A Total Mean [%]	Probe A	-17.82	-8.62	-3.11	-17.32	-15.79	-19.84
DGKL EQA		Mean	HP1/14	HP4/13	HP3/13	HP2/13	HP1/13
Measured Value [ng/mL]	Probe B	-	3101	3588	3243	3098	3149
Target Value [ng/mL]	Probe B	-	3422	4008	3360	4170	3618
Total Mean [ng/mL]	Probe B	-	3941	3864	3846	3857	3873
Δ B Targetvalue [%]	Probe B	-18.84	-9.38	-10.47	-3.47	-25.71	-12.97
Δ B Total Mean [%]	Probe B	-19.19	-21.32	-7.14	-15.67	-19.68	-18.70

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Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

REF	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
LOT	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός -Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
CONC	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
IVD	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κιτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
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