



User's Manual

IGFBP-3 ELISA

Enzymimmunoassay für die quantitative Bestimmung von
**humanem Insulin-like Growth Factor
Bindungs Protein-3**

Deutsch

Enzyme Immunoassay for quantitative Determination of
**human Insulin-like Growth Factor
Binding Protein-3**

English



DEM-DEE003A



96

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incubate at/ Inkubation bei/ Incuber à/ Incubare a/ incubar a/ Incubar a/ incubatie temperatuur/ Inkubation ved/ inkubation vid/ Inkubacja przy/ Inkubáció hőmérséklete/ Inkubácia pri/Inkubace při/ Инкубира се при/ Inkubatsioon temperatuuril/ Επώαση στους/ Incubare la/Inkubacija pri/ inkubaatiolämpötila



Mikrotiterplate/ Mikrotiterplatte/ plaque de microtitrage/ Piastra di microtitolazione/ Placa de microtitulación/ Placa de Microtitulação/ microtiterplaat/ Mikrotiterplade/ mikrotiterplatta/ microtiterplaat/ Plytka microtiter/ Mikrotiter lap/ Mikrotitračná podložka/ Mikrotitrační podložka/ Микротитърна плака/ Mikrotiterplaat/ Τρυβλίο μικροτιτλοδότησης/ Microplacă/ Mikrotitrska plošča/ Mikrotitruslevy



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Sample/ Probe / Echantillon/ campione/ Muestra/ Amostra/ monster/ Prøve/ prov/ Próbka/ Minta/ Vzorka/ Vzorek/ Проба/ Proov/ Δείγμα/ Probă/ Vzorec/ Näyte



Antibody and Enzyme Conjugate/ Antikörper und Enzym Konjugat/ anticorps conjugué et conjugué enzymatique/ Coniugato di anticorpo ed enzima/ Conjugado de anticuerpos y enzimas/ Conjugado Anticorpo-Enzima/ antilichaam- en enzymconjugaat/ Antistoffer og enzym-konjugat/ antikropp- och enzymkonjugat (antikropp och enzym, konjugat)/ Koniugat antyciał i enzymów/ Antitest és enzim páros/ Protílatkový a enzymatický konjugát/ Protílatkový a enzymatický konjugát/ Антитяло и ензим конюгат/ Antikehad ja ensüümi konjugaat/ Σύμπλοκο αντισώματος-ενζύμου/ Compuși din anticorpi și enzime/ Antitelesa in konjugat encima/ Vasta-aine ja entsyymi konjugaatti



Dilute in Buffer X/ Verdünnen in Puffer X/ Diluer dans le tampon X/ Diluire nel tampone X/ Diluir en tampón X/ Diluir no Tampão X/ verdunnen in buffer X/ Fortyndes i buffer X/ späđ i buffert X/ Rozcieńczenie w buforze X/ Hígítás X pufferben/ Riedit v pufru X/ Redit v pufru X/ Разреждане в буфер X/ Lahjendada puhvis X/ Αραιώστε σε ρυθμιστικό διάλυμα X/ Diluați în tamponul X/ Razrediti v pufru X/ laimennetaan x puskuriin

CAL X	A-E	Standard X/ Standard X/ Etalon X/ Standard X/ Estándar X/ Standard X/ standaard X/ Standard X/ standard X/ Standard X/ Standard X/ Štandard X/ Standard X/ Стандарт X/ Standard X/ Πρότυπο X/ Standard X/ Standardni X/ Kalibraattori X
Control	KS1/ KS2	Control Serum / Kontrollserum/ Contôle sérique/ Siero di controllo/ Suero de control/ Soro de Control/ controleserum/ Kontrolserum/ Kontrollserum/ Serum kontrolne/ Ellenőrző szérum/ Kontrolné sérum/ Kontrolní sérum/ Контролен сeрyм/ Kontrollseerum/ Ορός ελέγχου/Ser de control/ Kontrolni serum/ Kontrolli seerumi
WASHBUF 20x	WP	Washing Buffer Concentrate/ Waschpufferkonzentrat/ Tampon de lavage conc./ Tampone di lavaggio concentrato/ Tampón de lavado concentrado/ Tampão de Lavagem Concentrado/ wasbuffer, geconcentreerd/ Vaskebufferkonzentrat/ Vaskebufferkonzentrat/ tvättbuffertkonzentrat/ Bufor płukania koncentrat/ Mosópuffer koncentrátum/ Концентрат вымывачеho пуфра/ Концентрат на промивен буфер/ Pesupuhvi kontsentraat/ Συμπύκνωμα ρυθμιστικού διαλύματος έκπλυσης/ Concentrat pentru tamponul de spălare/ Koncentrat izpiralne puфра/ Pesuliuositiiviste
WASHBUF		Washing Buffer/ Waschpuffer/ Tampon de lavage/ Tampone di lavaggio/ Tampón de lavado/ Tampão de Lavagem/ wasbuffer/ Vaskebuffer/ tvättbuffert/ Bufor płukania/ Mosópuffer/ Вымывачи пуфр/ Вымывачи пуфр/ Промивен буфер/ Pesupuhver/ Ρυθμιστικό διάλυμα έκπλυσης/ Tampon pentru spălare / Izpiralni puфр/ Pesuliuos
SUBST TMB	S	Substrate/ Substrat/ Substrat/ Substrato/ Substrato/ Substrato/ substraat/ Substrat/ Substrat/ Substrat/ Szubsztrátum/ Substrát/ Substrát/ Субстрат Substraat/ Υπόστρωμα/ Substrat/ Substrat/ Substraattiliuos
H₂SO₄	SL	Stop Solution/ Stopp Lösung/ Stop Solution/ Soluzione di stop/ Stop Solución/ Solução Stop/ stopoplossing/ Stopopløsning/ Stopplösning/ Stop roztwór/ Megállító oldat/ Roztok na ukončenie/ Roztok pro ukončení/ Стопираци разтвор/ Stopp-lahus/ Διάλυμα διακοπής/ Soluție de oprire/ Stop raztopina/ Pysäytysliuos
TAPE		Cover Plate with sealing tape/ Platte abkleben/ Recouvrir la microplaque avec bande adhésive/ Coprire la piastra con nastro adesivo/ Cubrir la placa con una cinta adhesiva/ Cobrir a Placa com fita adesiva/ plaatje met tape afdekken/ Afdækningsplade med tape/ maskera platta/ Odkleić plytkę/ Tányér leragasztása/ Oblepiť podložku lepiacou páskou/ Olepit podložku lepící páskou/ Плака с лeнтa за запечатване/ Katta plaat isoleerkleplindiga/ Κολληστε το πλακίδιο με κολλητική ταινία/ Aoperiți placa cu o bandă adezivă/ Prelepiti ploščo/ Peitä mikrotitrauslevy oheisella teipillä
MEASURE		Measure plate within 30 min at 450 nm (Referencefilter ≥590nm)/Ausmessung innerhalb von 30 min bei 450 nm (Referenzfilter ≥ 590 nm)./ Measure lábsorbance en l'espace de 30 min à 450 nm avec ≥590nm longueur d'onde pour référence/Misurazione entro 30 min. a 450 nm (filtro di riferimento ≥ 590 nm)./ Medición de la placa dentro de los siguientes 30 min a 450 nm (filtro de referencia ≥ 590nm)/ Medir a placa dentro de 30 min a 450 nm (Filtro de referência ≥590nm)/ Binnen 30 minuten bij 450 nm meten (referentiefilter ≥ 590 nm)./ Mål plade i løbet af 30 min ved 450 nm (referencefilter ≥590nm)/ Mät inom 30 min vid 450 nm (referensfilter ≥ 590 nm)./ Pomiar w ciągu 30 min przy 450 nm (filtr odniesienia ≥ 590 nm)./ Ki mérése 30 percen belül 450 nm-nél (referenciaszűrő ≥ 590 nm)./ Merať 30 minút pri 450 nm/Měřit 30 minut při 450 nm/ Отчитане в рамките на 30 min при 450 nm (референтен филтър ≥ 590 nm)./ Mõõtmise 30 min jooksul 450 nm korral (võrdlusfilter ≥ 590 nm). Μέτρηση εντός 30 min στα 450 nm (φίλτρο αναφοράς ≥ 590 nm)./ Măsurare în decurs de 30 min la 450 nm (filtru de referință ≥ 590 nm)./ Izmerite ploščico v 30 min pri 450 nm (referenčni filter ≥590nm) / Mittaa 30 minuutin aikana 450 nm:ssä (referenssi suodatin ≥ 590 nm)
Literatur		Literature/ Literatur/ Bibliographie/ Letterario/ Bibliografía/ Literatura documentação/ literatuur/ Litteratur/ litteratur/ Literatura/ Irodalom/ Literatúra/ Literatura/ Литература/ Kirjandus/ Βιβλιογραφία/ Bibliografie/ literatura/ Lähdeluettelo
International Test description		International test description/ internationale Testanleitung/ description internationale de test/ Istruzioni per il test internazionali/ Descripción de ensayo internacional/ Descrição internacional do teste/ internationale testbeschrijving/ internationell testbeskrivning/ Opis testu międzynarodowego/ nemzetközi teszt-útmutató/ Medzinárodný návod k testu/ Mezinárodní návod k testu/ rahvusvaheline katse kirjeldus/ Διεθνείς οδηγίες για εργαστηριακές δοκιμές/ instrucțiuni internaționale pentru testare/ mednarodna navodila za preizkus/ Kansainvälinen käyttöohje
End		in all required wells/ in allen benötigten Vertiefungen/ dans tous les godets requis/ in tutti i pozzetti richiesti/ en todos los pozos requeridos/ em todos os tubos necessários/ in alle nodige putjes/ i alle nødvendige brønde/ i alla nödvändiga brunnar/ we wszystkich potrzebnych wgłębieniach/ minden szükséges forrásban/ vo všetkých potrebných miestach/ ve všech potřebných místech/ във всички необходими ямки/ köigis vajalikes süvendites/ σε όλες τις απαραίτητες κοιλότητες/ în toate cavitățile necesare/ v vseh zahtevanih vdolbinah/ kaikkiin tarvittaviin mikrotitrauslevyn syvennyksiin

Packungsbeilage Deutsch

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TECHNICAL FEATURES+APPLICATIONS

- ◆ Quantitative determination of IGFBP-3 without sample pretreatment
- ◆ Inter-Assay variation of 6.30% and Intra-Assay variation of 4.51%
- ◆ Sensitivity of 0.1 ng/ml
- ◆ Measures growth hormone (GH)-dependent IGFBP-3 Bindingprotein
- ◆ 2 Control Sera are provided for quality control purposes according GLP
- ◆ Stable serum levels due to absence of circadian variation
- ◆ Integrates the GH secretory state over days
- ◆ A single measurement is highly informative for diagnosis of GH deficiency or GH excess
- ◆ Ideal for the diagnosis of GH-deficiency in young children
- ◆ Small sample requirement, thus ideal for pediatric patients.

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, ... IGFBP-6 (1). Lately the discovery of a new IGFBP-7 has been discussed (2). 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 unique property to associate with an acid-labile non-binding subunit (ALS) after binding of either IGF-I or IGF-II (3-5). Most of the IGFBP-3 in plasma is present as the high molecular weight ternary complex, however, small amounts of free IGFBP-3 are also found (6,7).

The development of specific immunoassays for IGFBP-3, those also recognize the complete high molecular weight complex, provided new in-sights into its regulation (6-9). On the basis of these findings serum IGFBP-3 has proved 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 constant circadian levels (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.

So far, IGF-I serum levels have been widely used in screening for GH deficiency or acromegaly. However, several limitations are obvious:

1. The normal range of IGF-I is low in young children making discrimination of subnormal levels difficult at that age.
2. A considerable number of children of small stature have, despite normal GH secretion, IGF-I levels in the subnormal range. Therefore, the specificity and consequently the accuracy of the test for diagnosis of GH deficiency are limited.

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

1. No extraction step is required prior to measurement (as is still necessary in certain IGF-I assays) 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. A single measurement of the IGFBP-3 concentration is sufficient for the diagnosis of GH deficiency with high accuracy (7,18). 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.

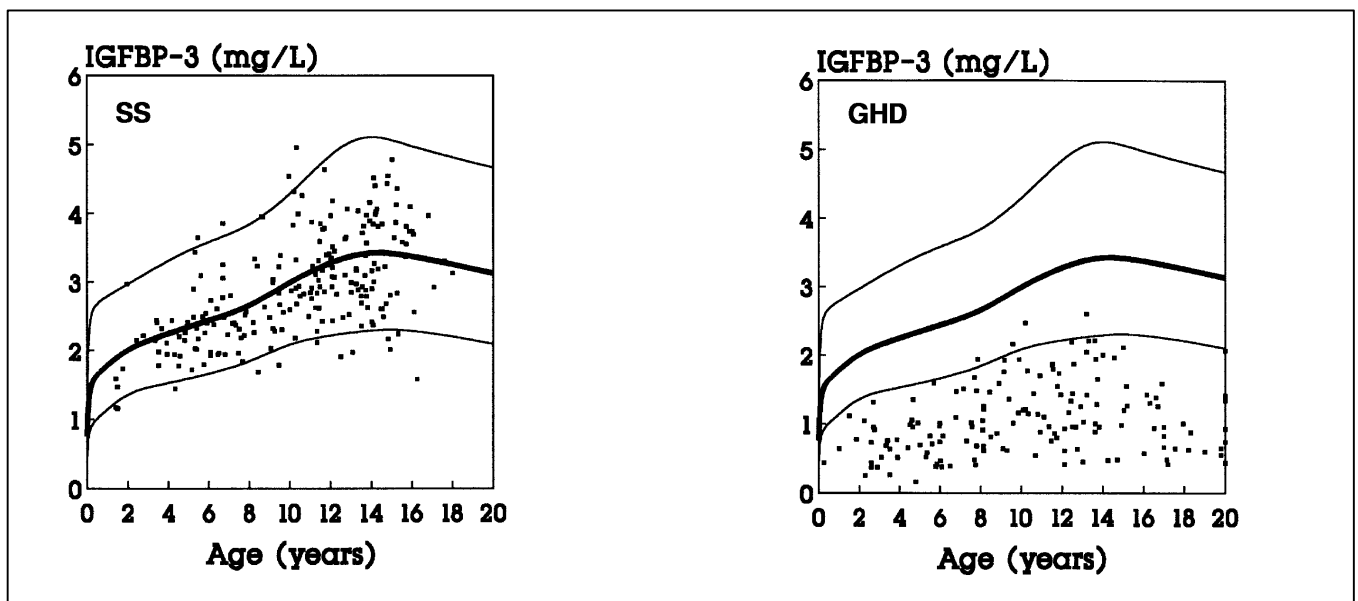


Figure 1: Serum IGFBP-3 levels in patients with short stature without GH deficiency (SS: constitutional delay of growth and adolescence, familial short stature, intra-uterine growth retardation) and in idiopathic or organic GH deficiency (GHD). The normal range is given by the 5th, 50th and 95th percentile.

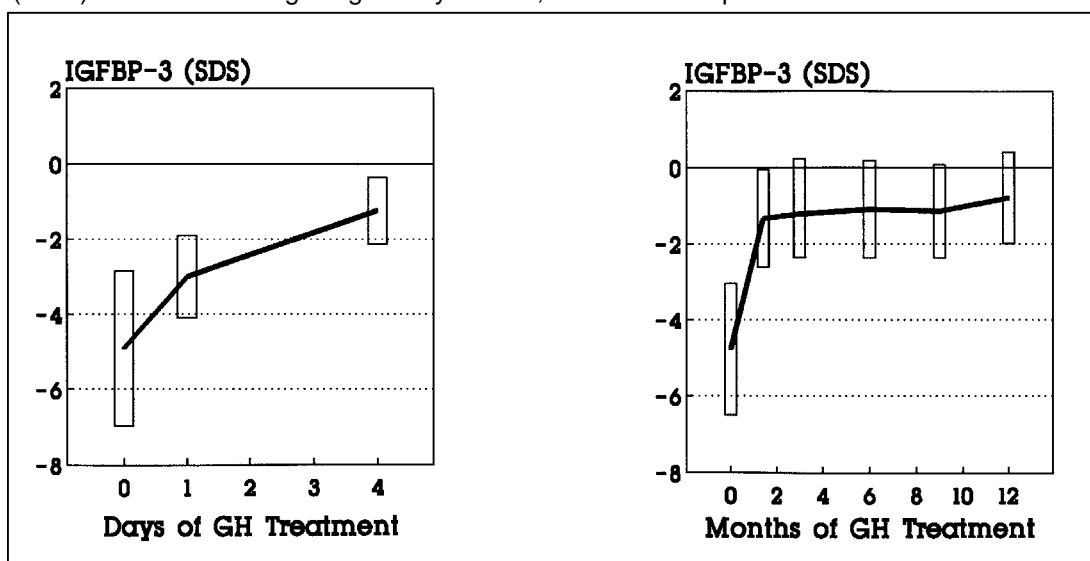
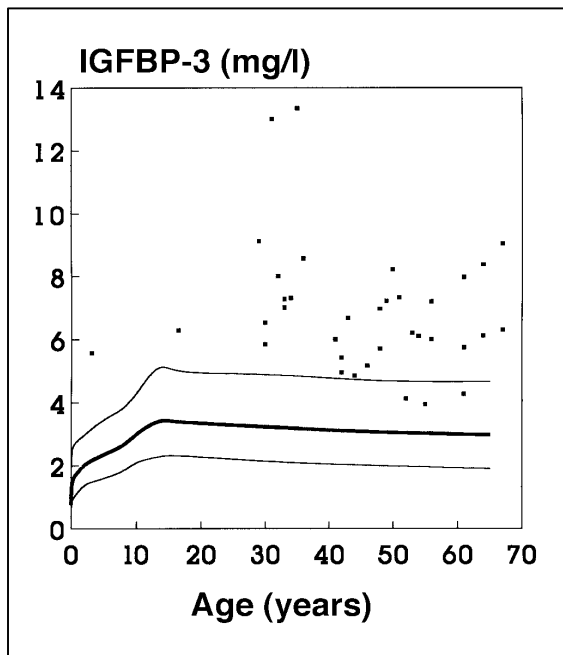


Figure 2: IGFBP-3 levels in GH deficient children before and during GH treatment. Because of the age-dependence, values are given as the mean of standard deviation scores (SDS).



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).

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

INTENDED USE

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

Its diagnostic value for GH deficiency screening is based on the high sensitivity and specificity of serum IGFBP-3 as a test for this diagnosis. States of GH excess may also be detected since IGFBP-3 levels are increased in that case. Due to its constant circadian concentration IGFBP-3 determination in a single blood sample may be sufficient as a screening test for these pathological situations prior to subjecting patients to further testing of GH secretion. IGFBP-3 determinations may also be suited for monitoring the efficacy of treatment and the patient's compliance in GH deficiency and acromegaly.

PERFORMANCE CHARACTERISTICS AND VALIDATION

The Demeditec ELISA for IGFBP-3 is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The IGFBP3 in the sample binds to the immobilized first antibody on the microtiter plate. In the following step, the biotinylated and Streptavidin-Peroxidase conjugated second specific anti-IGFBP-3-Antibody binds in turn to the immobilised IGFBP-3. In the closing substrate reaction the turn of the colour will be high specific catalysed, quantitatively depending on the IGFBP-3-level of the samples.

The standards of the ELISA DEE003A are **human IGFBP-3** in concentrations of **0.4; 2; 6; 15 and 30 ng/ml**.

Sensitivity

The **analytical sensitivity** of the ELISA DEE003A yields **0.1 ng/ml** (2 SD of zero standard in 18fold determination).

Table 1: Linearity

Dilution:	Sample 1 (recalculated, ng/ml)	Dilution:	Sample 2 (recalculated, ng/ml)
1:20	3250	1:20	3078
1:40	3489	1:40	3179
1:80	3181	1:80	3221
1:160	3167	1:160	3402
1:320	3013	1:320	3066
1:640	2936	1:640	2901
1:1280	2895	1:1280	3364
AV / 1SD / VC%	3133 / 205 / 6,54	AV / 1SD / VC%	3173 / 176 / 5.55

AV = Average Value , SD = Standard Deviation; VC = Coefficient of Variation
IGFBP-3 ELISA

DEE003A d/e 210709

The **Inter-** and **Intra-Assay** variation coefficients were found less than **6.30 %** and **4.51 %**. Exemplary determinations are shown in table 2 and table 3.

Table 2: Inter-Assay-Variation

	Mean Value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	2968	148	5.0
Sample 2	3334	210	6.30
Sample 3	4082	233	5.70

Table 3: Intra-Assay-Variation

	Mean Value (ng/ml)	Standard Deviation (ng/ml)	VC (%)
Sample 1	2968	148	5.0
Sample 2	2260	98.5	3.96
Sample 3	3699	167.0	4.51

Clinical validation was achieved by determination of IGFBP-3 levels in a large number of normal children and adults, normal short statured children without GH deficiency, girls with Ullrich-Turner Syndrome, children with Silver-Russell Syndrome, patients with GH deficiency, children with familial tall stature, Sotos-Syndrome, patients with acromegaly, children with premature thelarche and precocious puberty (Tab. 4; Abb. 1, 2, 3, 4 und 5).

SPECIMEN COLLECTION, PREPARATION AND STORAGE

Serum samples, Heparin-Plasma samples and Cerebrospinalfluid samples are suitable. A special external sample preparation prior to assay is not required. Results in Citrat- or EDTA-Plasma are about 15% reduced. Slight Hemolysis of the samples doesn't disturb the determination.

Samples should be handled as recommended in general: as fast as possible and chilled as soon as possible. In case there will be a longer period between the sample withdrawal and determination store the undiluted samples frozen -20°C or below in tightly closable plastic tubes. Avoid on principal repeated freeze-thaw cycles of serum/plasma (if required, please subaliquote) although IGFBP-3 levels were found to be unaffected by few cycles (5x) in our experiments.

The high sensitivity of the assays allows IGFBP-3 determinations in small sample volumes, which is limited by pipetting accuracy rather than the amount of IGFBP-3.

In most determinations (e.g. Serum- or Plasma samples and no extreme values expected) the dilution of **1:505 with Sample Buffer PP is suitable**, the respective covered range would be 0.2 to 15.15 mg/L. Where required, depending on the expected IGFBP-3-values, the dilution with **Sample Buffer PP** can be higher or lower. The IGFBP-3 concentrations maybe completely different in body fluids of human origin other than serum or in cell culture supernatants.

Suggestion for dilution protocol:

Pipette **1 ml Sample Buffer PP** (red colored) in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add **10 µl Serum-** or **Plasma** (dilution 1:101). Add 400 µl Sample Buffer **PP** in an other PE-/PP-tube and 100 µl of the thoroughly mixed first dilution (dilution 1:5). After mixing use **50 µl** of this 1:505 diluted solution within 1 hour **per determination** in the assay (pipetting control = red coloring of the solution in the wells).

REAGENTS PROVIDED

1)	MTP	Microtiter plate , ready for use: Microtiter plate with 96 wells, divided up in 12 strips with 8 wells separately breakable, coated with anti-human IGFBP-3 Antibody, packed in a laminate bag.
2)	CAL	Standards A-E , lyophilised, contain human IGFBP-3. Standard values are between 0.4 - 30 ng/ml (0.4, 2, 6, 15 and 30 ng/ml) IGFBP-3, Standards are reconstituted with 1 ml Sample Buffer PP each . Use 50 µl pro well in the assay.
3)	BUF VP	Dilution Buffer VP , 30 ml, ready for use, use 50 µl pro well in the assay.
4)	BUF PP	Sample Buffer PP , 120 ml, ready for use, red colored, please use for the reconstitution of Standards (A-E) and Controls and for dilution of Samples and Controls
5)	Control	Control Sera KS1 and KS2 , 250 µl, lyophilised, contain human Serum and should be reconstituted in each 250 µl Sample Buffer PP . The IGFBP-3 target values and the respective ranges are given on the vial labels. The dilution should be according to the dilution of the respected samples. Use 50 µl pro well in the assay.
6)	Ab CONJ	Antibody-HRP-Conjugate AK , 12 ml, ready for use , contains a mixture of biotinylated anti-human IGFBP-3 Antibody and HRP (Horseradish Peroxidase)-labelled Streptavidin. Use 100 µl pro well in the assay.
7)	WASHBUF 20x	Washing Buffer (WP) , 50 ml, 20X concentrated solution. Washing Buffer (WP) has to be diluted 1:20 with distilled or demineralised water before use (e.g. add the complete contents of the flask (50 ml) into a graduated flask and fill up with A.dest. to 1000 ml). Attention: After dilution the Washing Buffer is only 4 weeks stable, dilute only according to requirements.
8)	SUBST	Substrate (S) , 12 ml, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H ₂ O ₂ Tetramethylbenzidine.
9)	STOP	Stopping Solution (SL) , 12 ml, ready for use, 0.4 N acid solution, Caution acid!
10)		Sealing tape for covering of the microtiter plate, 2 x, adhesive.

MATERIALS REQUIRED, BUT NOT PROVIDED

Precision pipettes Micropipettes and multichannel pipettes with disposable plastic tips

Distilled or Deionized water for dilution of the Washing Buffer (WP)

Vortex-mixer

Device to aspirate the standards and the samples from the wells (recommended because of the potential danger of infection by human samples)

Reservoirs (disposable)

Plate washer

Calibrated Micro plate reader ("ELISA-Reader") with filter for 450 and 620nm (or ≥590 nm)

Technical Recommendations

The assay has to be conducted strictly according the test protocol herein.

Reagents with different lot numbers cannot be mixed. The microtiterplate and reagents are stable until the indicated expiry, if stored unopened and protected from sunlight at 2 – 8°C.

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.

Incubation at room temperature means: 20-25°C

Standards and Controls

For the reconstitution of the lyophilised components (Standards A - E and Control Sera KS1 & KS2) the kit Sample Buffer PP has to be used. 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.

The reconstituted standards and controls can be stored for 3 months at –20°C. Repeated freeze/thaw cycles have to be avoided. When using the standards anew, please thaw them rapidly but gently (no temperature rise over the room temperature and no powerful vortexing), 3 of these freezing-thawing cycles showed no influence on the assay.

Washing Buffer

The required volume of washing buffer is prepared by 1:20 dilution of the provided 20-fold concentrate with deionised water. The diluted Washing Buffer is stable for max. 4 weeks at 2-8°C.

Substrate Solution

The **Substrate Solution S**, stabilised H₂O₂-Tetramethylbencidine, is photosensitive – store and incubate in the dark.

Microtiterplate

Store the once unused microtiter strips and wells together with the desiccant in the tightly closed clip lock bag at 2-8°C use in the frame provided. The labelled expiry is not influenced in case of proper storage.

WARNINGS AND PRECAUTIONS

For in-vitro diagnostic use only. For professional use only.

Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. The Demeditec GmbH is not liable for any loss or harm caused by non-observance of the instructions, as far as no law withstands.

Temperature WILL affect the absorbance readings of the assay. However, values for the patient samples will not be affected.

Do not use expired reagents.

Use separate pipette tips for each sample, control and reagent to avoid cross contamination. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.

Caution: This kit contains material of human and/or animal origin.

Human Serum

Contained in following components: **Control Serum KS1 and KS2.**

The sources of human sera were tested by FDA recommended methods and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV) antibodies. No known test methods can offer total assurance of the absence of infectious agents; therefore all components and patient's specimens should be treated as potentially infectious.

Stop solution contains 0.4 N Acid

R36/38 Irritating to eyes and skin
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1 After contact with skin, wash immediately with plenty of water
S36/37 Wear suitable protective clothing and gloves

2-Methyl-4-Isothiazolin-3-one

contained in following components: **AK, VP, PP**

< 0.01% 2-Methyl-4-isothiazolin-3-one Solution

R34 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S36/37 Wear suitable protective clothing and gloves
S45 In case of accident or if you feel unwell seek medical advice

5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-Isothiazol-3-one

contained in following components: **AK, VP, WP, PP**

< 0.01% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-Isothiazol-3-one Solution

R36/38 Irritating to eyes and skin
R43 Sensibilisation through skin contact possible
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1 After contact with skin, wash immediately with plenty of water

TMB-Substrate (S) contains 3,3',5,5' Tetramethylbenzidine.

R20/21/R22	Harmful by inhalation, in contact with skin and if swallowed
R36/37/38	Irritating to eyes, respiratory system and skin
S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S28.1	After contact with skin, wash immediately with plenty of water
S36/37	Wear suitable protective clothing and gloves

General first aid procedures:

Skin contact: Wash affected area thoroughly with water. Discard 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: If swallowed, wash out mouth thoroughly with water. Immediately see a physician.

Do not eat, drink or smoke in these areas.

Never pipette the materials with the mouth.

Spilled material must be wiped off immediately and should become disinfected. Clean contaminated areas and equipment with a suitable detergent.

ASSAY PROCEDURE

NOTES: All determinations (Standards, Control Sera and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended. When performing the assay, the Standards, Control Sera and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, the **Antibody-POD-Conjugate AK**, the **Substrate Solution S** as well as the **Stop Solution SL** should be added to the plate in the same order and in the same time interval each, respectively.

- 1) Please pipette on before in **all needed wells 50 µl Dilution Buffer VP**.
- 2) Add **50 µl Sample Buffer PP** in positions A1/2.
- 3) Pipette in positions B1/2 **50 µl each Standard A (0.4 ng/ml)**, pipette in positions C1/2 **50 µl each Standard B (2 ng/ml)**, pipette in positions D1/2 **50 µl each Standard C (6 ng/ml)**, pipette in positions E1/2 **50 µl each Standard D (15 ng/ml)**, pipette in positions F1/2 **50 µl each Standard E (30 ng/ml)**.
To control the correct accomplishment **50 µl** of the 1:505 (or in respective dilution rate of the sample) in Sample Buffer **PP** diluted **Control Sera KS1 and KS2** can be pipetted in positions G1/2 and H1/H2.
Pipette **50 µl each** of the **diluted sample** (generally 1:505 diluted in Sample Buffer **PP**) in the rest of the wells, according to requirements. Please mix the dilutions immediately after sample addition and use within 60 minutes.
- 4) Cover the wells with the sealing tape and incubate the plate for **1 hour at room temperature** (without shaking).
- 5) After incubation aspirate the contents of the wells and wash the wells 5 times with **250 µl Washing Buffer WP**.
- 6) Following the last washing step pipette **100 µl** of the **Antibody-POD-Conjugate AK** in each well.
- 7) Cover the wells with the sealing tape and incubate **1 hour at room temperature** (without shaking).
- 8) After incubation wash the wells 5 times with **Washing Buffer WP** as described in step 5).
- 9) Pipette **100 µl of the TMB-Substrate solution S** in each well.
- 10) Incubate the plate for **30 Minutes in the dark at room temperature**.
- 11) After incubation pipette **100 µl Stop Solution SL** in each well.
- 12) Measure the absorbance **within 30 minutes at 450 nm** (**Reference filter ≥590 nm, e.g. 620 nm**).

CALCULATION OF RESULTS

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.20 and the absorbance of standard E should be greater than 1.00.

Samples, which yield higher absorbance values than **Standard E**, are beyond the standard curve, for reliable determinations such samples should be retested at a higher dilution.

Establishing 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** (MA) value for the blank from the duplicated determination (well A1/A2).
- 2) Subtract the mean absorbance (MA) of the blank from the mean absorbances of all other values.
- 3) Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis on semi-log paper (lin-log).
- 4) Recommendation: Calculation of the standard curve should be done by using a computer program because the curve is in general (without respective transformation) not ideally described by linear regression. **A higher-grade polynomial, or four parametric logistic (4-PL) curve fit or non-linear regression** are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).
- 5) The IGFBP-3 concentration in ng/ml of the samples can be calculated by **multiplication with the respective dilution factor**. Division by 1000 converts the values in µg/ml or, equal mg/Litre (Example: a measured value was 6 ng/ml, Sample was 1:505 diluted: $6 \times 505 = 3030$ ng/ml, or 3.03 µg/ml or 3.03 mg/L, according the requested unit).

EXPECTED NORMAL 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 4 by the percentiles (see Appendix). A graphic presentation is shown in Fig.4 and 5. It is recommended for each laboratory to establish its own normal range.

LIMITATIONS 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. In case of special interest in this physiological process, the Demeditec ELISA for **functional IGFBP-3** is available. The ELISA DEE004 enables to quantify the degree of **IGFBP-3 fragmentation** in samples.

Appendix /Anhang

Table 4: 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.

Tabelle 4: IGFBP-3 Serumkonzentrationen gesunder Probanden in Abhängigkeit vom Alter. Zwischen dem 7. und 17. Lebensjahr wurden die Daten nach Geschlecht getrennt ausgewertet, weil bei den Mädchen der Anstieg der Serumkonzentrationen während der Pubertät meistens 2 Jahre früher erfolgt.

Altersgruppe Age group	Percentiles / Perzentilen														
	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
Die Serumkonzentrationen sind in mg/L angegeben
week = Woche; months = Monate
y. = years; =Jahre

Determined with IGFBP-3 RIA (Blum et al. 1990)
Mit IGFBP-3-RIA gemessen (Blum et al. 1990)
The values above 70 years are extrapolated.
Die Werte für über 70-Jährige sind extrapoliert.

Serum conc. according to age

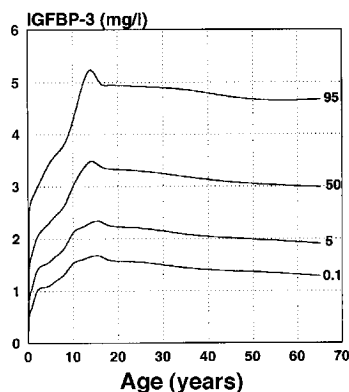


Fig. 4 Age-dependant normal values of IGFBP-3 (presented as 0.1., 5., 50., and 95. percentile)

Abb. 4: Altersabhängiger Normalbereich der IGFBP-3 Spiegel (0,1; 5.;50. und 90 Perzentile)

Children and adolescents

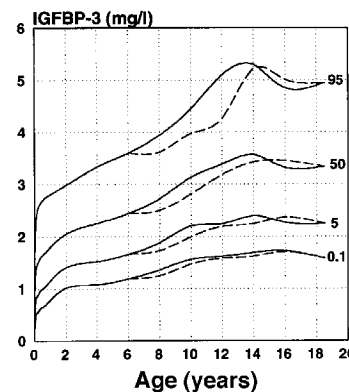


Fig. 5 Normal values of children and adolescents (girls — boys - - -)

Abb. 5: Normalbereich bei Kindern und Jugendlichen (Mädchen – Jungen- - -)

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KURZANLEITUNG – DEMEDITEC IGFBP-3 -ELISA DEE003A

Rekonstitution / Verdünnung von Reagenzien		
Standards A-E	Rekonstitution in Probenpuffer PP (rot)	je 1 ml
Kontrollseren KS1 & KS2	Rekonstitution in Probenpuffer PP (rot)	je 250 µl
Waschpuffer WP	verdünnen in A. dest. (z.B. die gesamte Menge von 50 ml im Standzylinder auf 1000 ml auffüllen)	1:20
Probenverdünnung bzw. Kontrollserumverdünnungen KS1&KS2: 1:505 in Probenpuffer PP (rot gefärbt), sofort mischen und in max. 60 min. verwenden. Davon 50 µl pro Bestimmung einsetzen (Pipettierkontrolle = Rotfärbung)		
Vor der Testdurchführung alle Reagenzien auf Raumtemperatur bringen.		

Testdurchführung Doppelbestimmung (Vorschlag):

Pipettieren	Reagenzien	Position
50 µl	Verdünnungspuffer VP	in alle benötigten Vertiefungen pipettieren
50 µl	Probenpuffer PP als Leerwert	A1 und A2
50 µl	Standard A (0.4 ng/ml)	B1 und B2
50 µl	Standard B (2 ng/ml)	C1 und C2
50 µl	Standard C (6 ng/ml)	D1 und D2
50 µl	Standard D (15 ng/ml)	E1 und E2
50 µl	Standard E (30 ng/ml)	F1 und F2
50 µl	Kontrollserum KS1	G1 und G2
50 µl	Kontrollserum KS2	H1 und H2
50 µl	Proben	in die Vertiefungen nach Bedarf

mischen, mit Klebefolie die Vertiefungen dicht abdecken.

Inkubation: 1 h bei RT

5x 250 µl	Absaugen und die Platte 5x mit je 250 µl Waschpuffer WP / Vertiefung waschen.	In jede Vertiefung
100 µl	Antikörper-POD-Konjugat AK	In jede Vertiefung

Inkubation: 1 h bei RT

5x 250 µl	Absaugen und die Platte 5x mit je 250 µl Waschpuffer WP / Vertiefung waschen.	In jede Vertiefung
100 µl	Substratlösung S	In jede Vertiefung

Inkubation: 30 min im Dunklen bei RT

100 µl	Stopplösung SL	In jede Vertiefung
Ausmessung innerhalb von 30 min bei 450 nm (≥590 nm Referenz),		

SUMMARY – DEMEDITEC IGFBP-3 ELISA DEE003A

Reconstitution / Dilution of Reagents		
Standards A-E	Reconstitution in Sample Buffer PP (red)	1 ml each
Control Serum KS1 & KS2	Reconstitution in Sample Buffer PP (red)	250 µl each
Washing Buffer WP	dilute in A. dest. (e.g. add the complete contents of the flask 50 ml into a graduated flask and fill with A.dest. to 1000 ml)	1:20
Sample Dilution + Control Sera KS1 & KS2: 1:505 in Sample Buffer PP (red colored), mix directly and use within max. 60 min. Use 50 µl per determination (pipetting control= red coloration)		
Before assay procedure bring all reagents to room temperature		

Proposal of Assay Procedure for Double Determination:

Pipette	Reagents	Well Positions
50 µl	Dilution Buffer VP	Pipette in <u>all</u> required number of wells
50 µl	Sample Buffer PP as Blank	A1 and A2
50 µl	Standard A (0.4 ng/ml)	B1 and B2
50 µl	Standard B (2 ng/ml)	C1 and C2
50 µl	Standard C (6 ng/ml)	D1 and D2
50 µl	Standard D (15 ng/ml)	E1 and E2
50 µl	Standard E (30 ng/ml)	F1 and F2
50 µl	Control Serum KS1	G1 and G2
50 µl	Control Serum KS2	H1 and G2
50 µl	Sample	Pipette sample in the rest of the wells according to requirements
Cover the wells with the sealing tape		

Incubation: 1 h at RT

5x 250 µl	Aspirate the contents of the wells and wash 5x with 250 µl each WP/well	each well
100 µl	Antibody-POD-Conjugate AK	each well

Incubation: 1 h at RT

5x 250 µl	Aspirate the contents of the wells and wash 5x with 250 µl each WP/well	each well
100 µl	Substrate Solution S	each well

Incubation: 30 min in the dark at RT

100 µl	Stop Solution SL	each well
Measure the absorbance within 30 min at 450 nm (≥590 nm Reference)		



CAL A-E	A -E	Rec in 1 ml PP	
Control	KS1&KS2	Rec in 250 µl PP	
WASHBUF 20x	WP		1:20 DILU A. dest.

Control	1:505 DILU PP
SPE	1:505 DILU PP
°C 20-25 °C	

50 µl	VP	A1 - End
50 µl	PP	A1/2
50 µl	CAL A (0.4 ng/ml)	B1/2
50 µl	CAL B (2 ng/ml)	C1/2
50 µl	CAL C (6 ng/ml)	D1/2
50 µl	CAL D (15 ng/ml)	E1/2
50 µl	CAL E (30 ng/ml)	F1/2
50 µl	CONTROL KS1 1:505 DILU PP	G1/2
50 µl	CONTROL KS2 1:505 DILU PP	H1/2
50 µl	SPE 1:505 DILU PP	
TAPE		

1 h **°C** 20-25

5x 250 µl	5x WASHBUF WP
100 µl	AbCONJ AK
TAPE	

1h **°C** 20-25

5x 250 µl	5x WASHBUF WP
100 µl	SUBST TMB S

0.5 h **°C** 20-25

100 µl	STOP SL
MEASURE	