Mouse/ Rat IGFBP-3 ELISA

Cat. No.: RMEE031R

TECHNICAL FEATURES + APPLICATIONS

m/rIGFBP-3 ELISA E031	96 Bestimmungen
RUO	For Research Use Only
Principle of the test	Enzyme-linked Immunoassay
Duration (Incubation period)	2.5 h
Antibodies	specific, goat anti-mouse/rat IGFBP-3 Antibody
Buffer	Ready for use and 20fold concentrate
Standard	7 Single standards: (0.39 - 25 ng/mL), native Mouse IGFBP-3
Assay Range	0.09 – 12625 ng/mL
Control	2 Control sera, lyophilized
Sample	Mouse- and Rat-Serum /Plasma Cell Culture Medium
Required sample volume	10 µL
Sample dilution	1:505
Analytical Sensitivity	Ø 0.09 ng/mL
Intra- / Interassay Variance	Ø ≤10 %

INTRODUCTION

Growth Hormone, Insulin-like Growth Factors and their binding proteins build up an endocrine system regulating not only longitudinal growth in humans but also influencing a broad variety of other physiological and pathophysiological processes like energy metabolism or tumor growth. Most effects of Growth Hormone (GH) are exerted by Insulin-like Growth Factors (IGF) mainly produced by the liver but also locally by specific tissues. The effects of IGF are also regulated, specific binding proteins (IGFBP 1-7) regulate bioavailability of IGF. After proteolytic cleavage of the binding proteins IGF is set free and able to bind to its receptor. The autophosphorylation of this thyrosine kinase receptor activates intra cellular signalling cascades. Some of these IGFBPs not only regulate the availability of IGF but also exert IGF-independent effects on cell physiology.

IGFBP-3 is the most abundant IGFBP in circulation and therefore of special relevance in regulation of IGF effects. This is reflected by the indicative value of serum IGFBP-3 concentration in diagnostics of growth disturbances. Regulation is effected e.g. through nourishing situation; Different diets for example affect the IGFBP-3 concentration (Bielohuby et al, 2010). IGFBP-3 has also been shown to be able to induce apoptosis, promote tumor growth and inhibit cellular migration and metastasis dependent on tissue and tumor stage.

Mouse / rat models for in vivo experiments are often used for studies of IGF-dependent and independent effects of IGFBP-3, particularly in the field of tumor research. For this purpose Mediagnost offers the E031 as a reliable and sensitive test system for the determination of IGFBP-3 in mouse and rat samples.

INTENDED USE

This enzyme immunoassay kit is suited for measuring IGFBP-3 in mouse and rat serum and plasma and in cell culture medium.

ASSAY PRINCIPLE

The Mediagnost m/rIGFBP-3 ELISA, E031 is a so-called sandwich-assay. It utilizes two different specific high affinity polyclonal antibodies for this protein. The IGFBP-3 in the samples binds quantitatively to the immobilized antibody. In the following step, the biotinylated antibody in turn binds IGFBP-3. After washing, a streptavidin-peroxidase-enzyme conjugate will be added, which will bind highly specific to the biotin of the antibody. 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.

WARNINGS AND PRECAUTIONS

For In Vitro Use only. For Professional use only.

The Mediagnost kit is suitable only for in vitro and not for internal use in humans and animals. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Mediagnost will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.

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

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

Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.

Animal serum: mouse / rat in the following components: KS1, KS2

Reagents AK, EK, VP, WP

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 (S)

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

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
P261	Avoid breathing dust/ fume/ gas/ mist/ 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 (SL)

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.

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.

SAMPLES

Sample type

Mouse and Rat Serum Plasma, In Heparin-Plasma samples the levels were found approx. 15% decreased. Further, cell culture medium was found to be suitable**.**

Specimen collection

Haemolytic reactions have to be avoided.

Requested sample volume: 10 µl serum.

Sample stability

- In firmly closable sample vials
- Storage at -20°C: min. 2 years
- Freeze/-thaw cycles: max. 3

It is recommended to store samples as soon as possible at least at 4°C. For any long time storage the sample has to be kept frozen at -20°C.

Sample dilution

Samples must be diluted prior to measurement. An extraction step is not required.

- Dilution: **1:505** with Dilution Buffer **VP**

  We recommend a dilution in 2 steps:

Pipette **1 mL** Dilution Buffer **VP** in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add **10 µL** Serum- or Plasma (dilution 1:101) and mix each tube immediately. Pipette **100 µL** of this dilution into another PE/PP vessel with **400 µL** of Dilution Buffer **VP** and mix immediately. This results in a final dilution of 1:505. After mixing, use 100 µL per assay in the assay within 1 hour of this solution

- After Mixing use 100 µL per assay in the assay within 1 hour of this solution.
- Where required, depending on the expected IGFBP-3-values, the dilution with **Dilution Buffer VP** can be higher or lower.

MATERIALS

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

MTP	**Microtiter plate,** ready for use, coated with goat anti-mouse IGFBP-3 Antibody, wells are separately breakable.	**(8x12) wells**
A-G	**Standards,** lyophilised (native Mouse-IGFBP-3), Concentrations are given on the vial labels and quality certificate.	**7 x 750 µL**
KS1	**Control Serum 1**, lyophilised, (Mouse Serum), Concentration is given **on the quality certificate.**	**1x 250 µL**
KS2	**Kontrollserum 2**, lyophilisiert, (Ratten Serum), Concentration is given **on the quality certificate.**	**1x 250 µL**
AK	**Antibody Conjugate**, ready for use, **Goat anti-mouse-IGFBP-3-Antibody, biotinylated.**	**1 x 12 mL**
EK	**Enzyme Conjugate EK**, contains HRP (Horseradish-Peroxidase)- labeled Streptavidin.	**1 x 12 mL**
VP	**Dilution Buffer**, ready for use. **Please shake before use.**	**1 x 125 mL**
WP	**Washing Buffer WP**, 20fold concentrated solution	**1 x 50 mL**
S	**Substrate S**, ready for use, horseradish-peroxidase (HRP)-substrate, stabilised Tetramethylbencidine.	**1 x 12 mL**
SL	**Stopping Solution SL**, ready for use, 0.2 M sulphuric acid.	**1 x 12 mL**
-	**Sealing Tape for covering the microtiter plate**	**3 x**
	**Instructions for use**	**1 x**
-	**Quality Control Certificate (QC-Certificate)**	**1 x**

Materials required, but not provided

- Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer **WP**

(A. dest.), 950 mL.

- Graduated cylinder for diluting Washing Buffer (WP)
- 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

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-G** and Control Sera **KS1 and KS2** must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Up to 3 of the freeze-thaw cycles did not influence the assay. The 1:20 diluted Washing Buffer **WP** is 4 weeks stable at 2-8°C

Preparation of reagents

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

Reconstitution

The Standards **A – G** and Control **KS1 and KS2** are reconstituted with the Dilution Buffer **VP**. It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.

Dilution

After reconstitution dilute the Control Sera **KS1 and KS2** with the Dilution Buffer **VP** in the same ratio (1:505) as the sample. The required volume of Washing Buffer **WP** is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest.

Incubation

Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate Solution **S**, stabilised H_2O_2-Tetramethylbencidine, is photosensitive–store and incubation in the dark.

Assay Procedure

When performing the assay, Blank, Standards **A-G**, Control Serum **KS** and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody Conjugate **AK,** Enzyme Conjugate **EK** as well as the succeeding Substrate Solution **S** should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution **SL** should be added to the plate in the same order as Substrate Solution **S**.

All determinations (Blank, Standards **A-G**, Control Serum KS and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

Shaking

The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must be adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/ or false values, excessive shaking may result in high optical densities and/ or false values.

Washing

Proper washing is of basic **importance** for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems.

All washing must be performed with the provided Washing Buffer **WP** diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least.

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

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

Manual washing is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

ASSAY PROCEDURE

Reagent preparation		Reconstitution	Dilution
A-G	**Standards**	in **750 µL** Dilution Buffer **VP**	-
KS1	**Control Serum 1**	in **250 µL** Dilution Buffer **VP**	**1:505** with Dilution Buffer **VP**
KS2	**Control Serum 2**	in **250 µL** Dilution Buffer **VP**	**1:505** with Dilution Buffer **VP**
WP	**Waschpuffer**	-	**1:20** with **Aqua dest.**

Dilute **Samples** with Dilution Buffer **VP 1:505**

Before assay procedure bring all **reagents** to **room temperature (20°C- 25°C)**

Assay Procedure in Double Determination:

Pipette	Reagents	Position
100 µL	Dilution Buffer **VP** (Blank)	A1/A2
100 µL	Standard **A** (**0.39 ng/mL**)	B1/B2
100 µL	Standard **B** (**0.78 ng/mL**)	C1/C2
100 µL	Standard **C** (**1.56 ng/mL**)	D1/D2
100 µL	Standard **D** (**3.13 ng/mL**)	E1/E2
100 µL	Standard **E** (**6.25 ng/mL**)	F1/F2
100 µL	Standard **F** (**12.5 ng/mL**)	G1/G2
100 µL	Standard **G** (**25 ng/mL**)	H1/H2
100 µL	Control Serum **KS 1** (1:505 diluted)	A3/A4
100 µL	Control Serum **KS 2** (1:505 diluted)	B3/B4
100 µL	Sample (1:505 diluted)	In the rest of the wells according to the requirements.
Cover the wells with the sealing tape.		
Incubation: 1 h at 20-25°C, 350 rpm		
5x 300 µL	Aspirate the contents of the wells and wash **5x** with **300 µL** each **WP**/**well**	Each well
100 µL	Antibody Conjugat **AK**	Each well
Cover the wells with the sealing tape		
Incubation: 1 h bei 20-25°C, 350 rpm		
5x 300 µL	Aspirate the contents of the wells and wash **5x** with **300 µL** each **WP**/**well**	Each well
100 µL	Enzyme Conjugate **EK**	Each well
Cover the wells with the sealing tape		
Incubation: 15 min at 20-25°C, 350 rpm		
5x 300 µL	Aspirate the contents of the wells and wash **5x** with **300 µL** each **WP**/**well**	Each well
100 µL	Substrate Solution **S**	Each well
Substrat S Incubation: 15 Minutes in the dark at RT		
100 µL	Stop Solution **SL**	Each well
Measure the absorbance within 30 min at **450 nm** (≥590 nm Reference)		

CALCULATION OF RESULTS

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 G should be above 1.00.

Samples, which yield higher absorbance values than **Standard G**, 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 concentration of **mIGFBP-3**:

Standard	A	B	C	D	E	F	G
ng/mL	0.39	0.78	1.56	3.13	6.25	12.5	25

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 values.

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** 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 5.760 ng/mL, Sample was 1:505 diluted: 5.760 x 505= 2909 ng/mL, or 2.909 µg/mL equal to 2.909 mg/L)

Example of a typical standard curve

Standard	Blank	A	B	C	D	E	F	G
ng/mL	0	0.39	0.78	1.56	3.13	6.25	12.5	25
OD (450-620 nm)	0.00	0.048	0.101	0.202	0.412	0.815	1.484	2.438

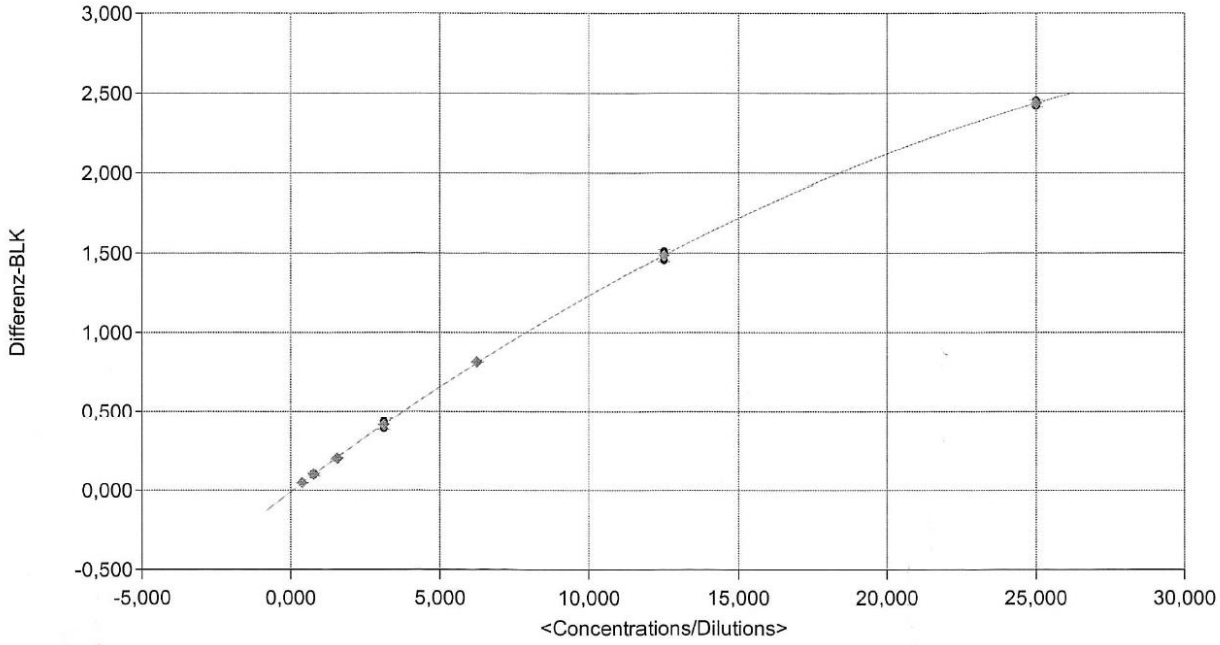

Fig. 1: Exemplary Standard Curve with a polynomial 3rd degree as curve fit.

The exemplary shown standard curve in Fig.1 **cannot be used** for calculation of your test results. You have to establish a standard curve for each test you conduct!

Exemplary calculation of the IGFBP-3 concentration of a diluted sample:

OD 450 nm

Measured extinction (mean value) of your sample	0.749
Measured extinction of the blank (mean value)	0.000

Your **measurement program** will calculate the IGFBP-3 concentration of the 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 3rd degree).

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

$0.749 = 3{,}62^{-6} \times X^3 + -0.00188 \times X^2 + 0.143 \times X - 0.011$

$5.760 = X$

Multiplication by dilution factor (1:505) gives the IGFBP-3 concentration of the sample with

2909 ng/mL

PERFORMANCE CHARACTERISTICS

Calibration

The Mediagnost E031 was recalibrated on a highly purified eukaryotic expressed recombinant rat IGFBP-3 preparation. The previous calibration on a recombinant mouse IGFBP-3 preparation of lower purity and with a partly different amino acid sequence resulted approx. by a factor of 10 lower values. If desired for comparison purposes, a conversion from or to old values can be carried out by a factor of 10. Measured values with **previous calibration**, e.g. 300 ng/mL correspond to a **new calibration** of 3000 ng/mL. It is also possible to calculate the previous calibration by dividing by 10.

Analytical Sensitivity

The **analytical Sensitivity** was assessed by 21-fold determination of the blank and calculating the theoretical concentration of the blank +2SD. The analytical sensitivity of the E031 is **0.09 ng/mL**

Precision

The **Inter**- and **Intra-Assay** variation coefficients were on average **≤10%.** Exemplary determinations are shown in table 1 and table 2.

Tabelle 1 Inter-Assay-Variation (n=26 or 15)

	Mean Value (ng/mL)	Standard Deviation (ng/mL)	VC(%)
Sample 1	4836	384	7,94
Sample 2	2625	269	9,43

Tabelle 2 Intra-Assay-Variation (n=13)

	Mean Value (ng/mL)	Standard Deviation (ng/mL)	VC (%)
Sample 1	3286	124	3,76
Sample 2	1529	123	8,02

Linearity

Table 3: Linearity (results of 2 different mouse sera)

Dilution:	Sample 1 (recalculated, ng/mL)	Sample 2 (recalculated, ng/mL)
1:100	3518	3676
1:200	3691	4145
1:400	3845	4234
1:800	3813	4110
1:1600	3792	4219
1:3200	3861	4557
AV/ SD/ VC%	3753/ 129/ 3.46	4157/ 284/ 6.83

AV = Average Value, SD = Standard Deviation; VC = Coefficient of Variation

Species Cross-Reactivity

Serum of the cited species were used as diluted samples in this assay system.
No cross reactivity was detected for:
Rabbit, Cat, Chicken, Guinea pig, Goat, Sheep, Horse, Donkey, Pig, Dog, Bovine.

Cross reactivity with recombinant human eukaryotic expressed IGFBP-3 (1 µg/mL): 0.06%

Recovery in Cell Culture Medium

The recovery of recombinant mouse IGFBP-3 in **cell culture medium** DMEM was found to be 89.4%, and, in DMEM incl. 5% FCS 92.6%. Therefore, cell culture medium seems to be suitable as sample matrix.

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INTERNATIONAL ASSAY DESCRIPTION

A-G	**STD**	**Rec in** 750 µL **BUF VP**	**100 µL**
KS1	**Control**	**Rec in** 250 µL **BUF VP**	**100 µL**
KS2	**Control**	**Rec in** 250 µL **BUF VP**	**100 µL**
WP	**WASHBUF 20x**	-	**1:20 DILU A. dest.**
SPE + **Control** **1:505** **DILU BUF VP**	↔	**max. 1 h**	**100 µL**
°C 20-25°C			

100 µL	**BUF VP**	A1/2
100 µL	**STD A (0.39 ng/mL)**	B1/2
100 µL	**STD B (0.78 ng/mL)**	C1/2
100 µL	**STD C (1.56 ng/mL)**	D1/2
100 µL	**STD D (3.13 ng/mL)**	E1/2
100 µL	**STD E (6.25 ng/mL)**	F1/2
100 µL	**STD F (12.5 ng/mL)**	G1/2
100 µL	**STD G (25 ng/mL)**	H1/2
100 µL	**CONTROL KS1 1:505 DILU BUF VP**	A3/4
100 µL	**CONTROL KS2 1:505 DILU BUF VP**	B3/4
100 µL	**SPE 1:505 DILU BUF VP**	
	TAPE	
	1 h °C 20-25 ↔ **350 rpm**	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**Ab AK**	
	TAPE	
	1 h °C 20-25 ↔ **350 rpm**	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**CONJ EK**	
	TAPE	
	15 min °C 20-25 ↔ **350 rpm**	
5x 300 µL	**5x WASHBUF WP**	
100 µL	**SUBST TMB S**	
	15 min °C 20-25	
100 µL	**H_2SO_4 SL**	
	MEASURE	

ASSAY PROCEDURE

Reagent preparation		Reconstitution	Dilution
A-G	**Standards**	in **750 µL** Dilution Buffer **VP**	-
KS1	**Control Serum 1**	in **250 µL** Dilution Buffer **VP**	**1:505** with Dilution Buffer **VP**
KS2	**Control Serum 2**	in **250 µL** Dilution Buffer **VP**	**1:505** with Dilution Buffer **VP**
WP	**Waschpuffer**	-	**1:20** with **Aqua dest.**

Dilute **Samples** with Dilution Buffer **VP 1:505**

Before assay procedure bring all **reagents** to **room temperature (20°C- 25°C)**

Assay Procedure in Double Determination:

Pipette	Reagents	Position
100 µL	Dilution Buffer **VP** (Blank)	A1/A2
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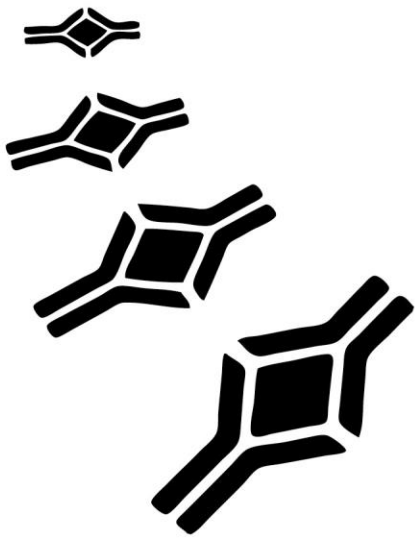
100 µL	Control Serum **KS 2** (1:505 diluted)	B3/B4
100 µL	Sample (1:505 diluted)	In the rest of the wells according to the requirements.
Cover the wells with the sealing tape.		
Incubation: 1 h at 20-25°C, 350 rpm		
5x 300 µL	Aspirate the contents of the wells and wash **5x** with **300 µL** each **WP**/**well**	Each well
100 µL	Antibody Conjugat **AK**	Each well
Cover the wells with the sealing tape		
Incubation: 1 h bei 20-25°C, 350 rpm		
5x 300 µL	Aspirate the contents of the wells and wash **5x** with **300 µL** each **WP**/**well**	Each well
100 µL	Enzyme Conjugate **EK**	Each well
Cover the wells with the sealing tape		
Incubation: 15 min at 20-25°C, 350 rpm		
5x 300 µL	Aspirate the contents of the wells and wash **5x** with **300 µL** each **WP**/**well**	Each well
100 µL	Substrate Solution **S**	Each well
Substrat S Incubation: 15 Minutes in the dark at RT		
100 µL	Stop Solution **SL**	Each well
Measure the absorbance within 30 min at **450 nm** (≥590 nm Reference)		



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