BioVendor
Research
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Human IGFBP-1 ELISA

Cat. No.: RMEE01

IVD CE

TECHNICAL FEATURES+APPLICATIONS

- **Quantitative determination of IGFBP-1** in serum and in other body fluids, like e.g. amniotic fluid, milk, urine or saliva etc., and in cell culture media.
- extremely high **analytical sensitivity of 0.02 ng/ml**
- Inter-Assay variation of 7,4% and Intra-Assay variation of 6,8%
- results available in **only 1.75 h incubation time**

INTRODUCTION

The Insulin-like Growth Factors I and – II are free in body fluids and tissues but are bound to specific binding proteins. Until today seven different binding proteins (IGFBP-1 to –7) can be differentiated additionally several IGFBP-related proteins have also been detected. Bioavailability of IGF is regulated by these IGFBPs or better their proteolytic cleavage which reduces affinity to IGF. But the IGFBPs as well as their proteolytic fragments can also exert IGF-independent effects, like influencing cell migration or proliferation.

IGFBP-1 (Placental Protein 12) consists of 234 aminoacids and has a molecular weight of approximately 25kDa. The coding DNA region is located on chromosome 7 [1, 2]. IGFBP-1 is mainly synthesized by foetal and adult liver tissue and decidual endometrium. Intensity of Expression varies enduring menstruation with a maximal expression in the late secretory phase [3, 4]. Further IGFBP-1 expression seems to be regulated by Insulin concentration, with Insulin inhibiting the expression. Insulin regulation results in diurnal fluctuations of up to factor 10 [5] . IGFBP-1 is posttranslational modificated by phosphorylation of serine residues 101, 119 and 169. Phosphorylation has physiological relevance as it increases affinity of IGFBP-1 to IGF. In adult humans phosphorylated IGFBP-1 of the liver is the predominant form in circulation. IGFBP-1 produced by endometrial tissue is significantly less phosphorylated than the liver originated form [6].

In pregnancy IGFBP-1 maternal serum concentration increases significantly with maximal values in the second trimester or 22-23 week of gestation (75.8 ng/ml) [5] and decreases slowly until term. IGFBP-1 concentration are not only increased in maternal but also in foetal serum and with extremely high concentrations in amnion fluid. Here concentration can reach more than the 1000-fold of serum values [7]. Long-term changes of serum IGFBP-1 concentration can also be found in amnion fluid: IGFBP-1 level of the child decreases after birth until it reaches the low steady-state level of puberty and adulthood [8, 9].

Short term IGFBP-1 serum concentration is strongly influenced by nutrition level and therewith by insulin. Decreasing IGFBP-1 levels can be found enduring fasting or in diabetes; IGFBP-1 levels increase in case of intensive exercises [10-12].

Relevance of serum and amnion IGFBP-1 in diagnostics has been investigated in several areas. A diagnostic value was assigned for trisomy 18, intrauterine growth retardation, endometrial tumors and pre-eclampsia [14].

Thoroughly investigated was the diagnostic value in insulin resistance and pre-term rupture of the membrane and specially in the second field a significant diagnostic value could be demonstrated.

Energy metabolism

Based on the influence of Insulin on IGFBP-1 serum concentrations IGFBP-1 is said to be a possible marker for insulin resistance. Because measurement of IGFBP-1 is much easier facilitated than Glucose – uptake rate this would simplify diagnosis of insulin resistance.

In a small study Maddux et al were able to demonstrate with 23 non-diabetic patients, that IGFBP-1 serum concentration correlated very well with Glucose-uptake rate, even better than the HOMA index does [13].

Pregnancy

In pregnancy a significant difference in IGFBP-1 serum concentration of healthy pregnant and diabetic and pre-eclamptic women was found (102,8 vs. 203,71 or 281,09 ng/ml respectively) [15].

Also the evaluation of IGFBP-1 as marker for membrane rupture showed a high specificity (97%) and sensitivity (75%) of IGFBP-1 in vaginal/cervical secrets. In case of intact membrane IGFBP-1 concentration was < 90ng/ml in the secretion. Enduring 8 hours after spontaneous or induced membrane rupture IGFBP-1 values increased significantly with a median concentration of 1900 ng/ml. In this study IGFBP-1 concentrations von >100ng/ml were set as threshold for detection of amnion fluid and therewith diagnosis of membrane rupture [16]. A positive predictive value of 97% clearly shows that IGFBP-1 is a suitable marker for premature membrane rupture [17] .

INTENDED USE

This enzyme immunoassay kit is suited for measuring IGFBP-1 in human serum or Heparin-plasma or in other body fluids, for example amnion fluid, mother milk, urine or saliva, as for diagnostic and scientific purposes. It is also suited to quantitate IGFBP-1 in cell culture media.

PERFORMANCE CHARACTERISTICS and Validation

The ELISA for IGFBP-1 RMEE01 is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The IGFBP-1 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-1-Antibody binds in turn to the immobilised IGFBP-1. Finally, the bound peroxidase catalyses the substrate reaction resulting in a colored product. Therefore colour intensity is highly specific and quantitatively depending on the IGFBP-1-level of the samples.

The standards of the ELISA RMEE01 are **native human IGFBP-1** in concentrations of **0, 0.1, 0.5, 1, 2, 4 and 8 ng/ml**.

The **analytical sensitivity** of the ELISA RMEE01 yields **0.02 ng/ml** (equal to **2 pg per well**; 2 SD of zero standard in 22fold determination).

The determination of IGFBP-1 with ELISA RMEE01 is over a very wide range authentic in dilution. The **linearity of serum dilutions** is over a wide range **excellent** (table 1).

Table 1: Linearity of Dilution (typical results of 2 different sera)

Dilution:	**sample 1** (re-calculated, ng/ml)	**Dilution:**	**sample 2** (re-calculated, ng/ml)
1:2.5	14.38	1:2.5	16.81
1:5	14.22	1:5	15.51
1:10	13.42	1:10	16.22
1:20	13.81	1:20	14.45
1:40	13.11	1:40	15.12
1 :80	12.52	1 :80	13.43
1 :160	14.65	1 :160	15.95
AV / 1SD / CV%	**13.73** / 0.76 / **5.53**	**AV / 1SD / CV%**	**15.36** / 1.14 / **7.44**

AV = average value, SD = standard deviation, CV = coefficient of variation

The **recovery** of native IGFBP-1 in different sample matrices is listed in table 4 (page 13).

The measured **cross reactivity** for recombinant IGFBP-2 as well as IGFBP-3 was found to be negligible, measured in 500 ng/ml each, **less than 0.0015%** were quantitated.

The **Inter**- and **Intra-Assay** coefficients of variation were found less than **7.4% and 6.8%**. Exemplary determinations are shown in table 2 and table 3.

Table 2: Inter-Assay-Variation

	Average Value (ng/ml)	Standard Deviation (ng/ml)	Coefficient of Variation (%)
Sample 1	2.31	0.12	5.23
Sample 2	18.41	1.36	7.36
Sample 3	32.79	2.22	6.75

Table 3: Intra-Assay-Variation

	Average Value (ng/ml)	Standard Deviation (ng/ml)	Coefficient of Variation (%)
Sample 1	1.45	0.08	5.87
Sample 2	20.64	1.29	6.23
Sample 3	162.99	11.09	6.81

The comparison of IGFBP-1 determinations of 35 sera from healthy adults with the ELISA RMEE01 and another commercially available ELISA yields a very good accordance of absolute concentrations by a **very high Correlation**: y= 1.15x + 0.12; **r^2= 0.94**, the comparison with a further commercial ELISA yields, at a likewise **very high Correlation**: y= 3.33x + 3.0; **r^2= 0,90**, measured values of approx. one third of the respective concentrations.

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

Serum samples, EDTA- and Heparin-Plasma samples are suitable. A special external sample preparation prior to assay is not required. Results in Citrate-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-1 levels were found to be unaffected by few cycles(3x) in our experiments.

In most determinations (e.g. Serum- or Plasma samples and no extreme values expected, see table 4 for further details) the dilution of **1:16 with Dilution Buffer VP is suitable**, the respective covered range would be 0 to 128 ng/ml.

Suggestion for dilution protocol:

Pipette 300 µl **Dilution Buffer VP** in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add **20 µl Serum**- or **Plasma** (dilution 1:16) and mix each tube **immediately**. After mixing use **50 µl** of this solution within 1 hour **per determination** in the assay.

Where required, depending on the expected IGFBP-1-values, the dilution with Dilution Buffer VP can be higher or lower (at least however 1:2.5). The IGFBP-1 concentrations maybe completely different in body fluids of human origin other than serum or in cell culture supernatants. Examples as well as dilution recommendations are given in table 4.

Table 4: Sample matrices, recovery and dilution recommendation

Samples	Concentration IGFBP-1 (ng/ml)	Recovery of added IGFBP-1	Recommended Dilution as Sample in RMEE01
Amniotic Fluid	8,140.0 16,450.0	n.d.	1:8,000
Mother Milk	5.12 20.2	91% (at 1:10 dil.) n.d.	1:10
Urine	0.07	89,8% (at 1:2.5 dil.)	1:2.5
Saliva	< 0.02 ng/ml	62,5% (at 1:2.5 dil.)	at least 1:2.5
Bronchial Lavage	< 0.02 ng/ml	100% (at 1:2.5 dil.)	1:2.5
Sputum	< 0.02 ng/ml	100% (at 1:20 dil.)	1:20
Serumpool	0.57	105.1% (at 1:16 dil.)	1:16 (general recommendation)
Pregnancy sera	n.d.	n.d.	1:25
Cell Culture Media	individually different	94,5% (at 1:5 dil.)	individually different at least 1:5

n.d.= not determined

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-1 Antibody, packed in a laminate bag.
2)	CAL	**Standards A-G**, lyophilised, contain native human IGFBP-1. Standard values are between 0 – 8 ng/ml (0, 0.1, 0.5, 1, 2, 4 and 8 ng/ml) IGFBP-1, Standards are **reconstituted with 500 µl Dilution Buffer VP each**. Use 50 µl pro well in the assay.
3)	BUF X	**Dilution Buffer VP**, 125 ml, ready for use, please use for dilution of samples, control and standards.
4)	Control	**Control Sera KS1 and KS2**, 250 µl, lyophilised, contain human Serum and should be **reconstituted in 250 µl Dilution Buffer VP each**. The IGFBP-1 target values and the respective ranges are given on the vial label. The dilutions should be according to the dilution of the respected samples. Use 50 µl pro well in the assay.
5)	Ab	**Antibody Conjugate AK**, 6 ml, contains biotinylated anti-human IGFBP-1 Antibody. Use 50 µl pro well in the assay.
	CONJ	**Enzyme Conjugate EK**, 12 ml, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin. Ready for use. Use 100 µl pro well in the assay.
6)	WASHBUF 20x	**Washing Buffer (WP),** 50 ml, 20 X concentrated solution. Dilute 1:20 with Aqua dest. The 1:20 diluted Washing Buffer **WP** is only limited stable. Please dilute only according to daily requirements.
7)	SUBST	**Substrate (S),** 12 ml, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbencidine. Use 100 µl pro well in the assay
8)	H_2SO_4	**Stopping Solution (SL),** 12 ml, ready for use, 0.2 M sulphuric acid, Caution acid! Use 100 µl pro well in the assay
9)		Sealing tape for covering of the microtiter plate, 2 x, adhesive.

MATERIALS REQUIRED BUT NOT PROVIDED

Precision pipettes (100 and 200µl) 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)

Timer (120 min. range)

Reservoirs (disposable)

Plate washer and plate shaker (recommended)

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

Foil welding device for laminate bags (recommended)

REAGENT PREPARATION

In conducting the assay, follow strictly the test protocol. Room temperature incubation means: Incubation at 20 - 25°C.

Reagents with different lot numbers should not be mixed. The microtiter plate and all reagents are stable unopened until the expiry date, if stored in the dark at 2° - 8°C (see label).

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

Use the **Dilution Buffer VP** for the dilution of **Samples, Standards** and **Controls.**

The shelf life of the components after opening is not affected, if used appropriately. Store the unused seal stripes of the microtiter plate together with the desiccant at 2-8°C. Reconstituted Components (**Standards A – G** and **Control Sera KS1 and KS2**) should be stored at -20°C (or below). Freezing extends the expiry at least 2 months. 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.

The 1:20 diluted Washing Buffer **WP** is only limited stable. Please dilute only according to daily requirements.
Before use, all kit components should be brought to room temperature. **Precipitates, possible in buffers, should be dissolved before use through mixing and warming**.

The **Substrate Solution S**, stabilised H_2O_2-Tetramethylbencidine, is photosensitive – store and incubate in the dark.

When performing the assay, the Standards **A-G,** Control Sera **KS1** and **KS2** and the samples should be pipetted as fast as possible (e.g., 15 minutes). To avoid distortions due to differences in incubation times the 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. **Stop Solution SL** should be added to the plate in the same order as the Substrate Solution **S.**

STORAGE CONDITIONS

The microtiter plate wells and all undiluted reagents are stable until the expiry date if stored in the dark at 2-8°C.
Store the unused seal strips and microtiter wells together with the desiccant at 2° to 8°C.

The Substrate Solution (S), stabilised H_2O_2-Tetramethylbencidine, is photosensitive – store and incubate in the dark.

Reconstituted components should be stored at 2-8°C for up to 1 week. If longer storage time is needed, store the components frozen at -20°C or below. Freezing extends the expiry at least 2 months. Avoid repeated freeze-thaw cycles. In case you plan to perform multiple independent determinations over a longer period with one kit, you should aliquot the components prior to freezing into suitable smaller volumes. This is strongly recommended.

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.

Before use, all kit components should be brought **to room temperature at 20 - 25°C**. Precipitates in buffers should be dissolved before use by thorough mixing and warming. **Temperature WILL affect the absorbance** readings of the assay. However, values for the patient samples will not be affected.

Do not mix reagents of different lots. Do not use expired reagents.

The microplate contains snap-off strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.

Caution: This kit contains material of human and/or animal origin. Source human serum for the Control Sera provided in this kit was 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) antibody. 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 0.2 M Sulphuric Acid (H_2SO_4)

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.

Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step. 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.

ProClin 950

Following components contain ProCline 950 : **AK, EK, VP**

< 0,1% 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

Kathon CG

Following components contain Kathon CG **AK, EK, VP, WP**

< 0,1% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one und 2-methyl-2H-isothiazol-3-one

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 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 Serum and the samples should be pipette as fast as possible (e.g., <15 minutes). To avoid distortions due to differences in incubation times, **Enzyme Conjugate EK** as well as the following **Substrate Solution S** should be added to the plate in the same order and in the same time interval as the samples. **Stop Solution SL** should be added to the plate in the same order as the Substrate Solution. Before beginning the test procedure bring all reagents to room temperature.

IMPORTANT: Please leave the wells A1/A2 until addition of the **Substrate Solution**, step 9, empty.

1) Please pipette in all needed wells**, <u>except A1/A2</u>, 50 µl Antibody Conjugate AK.**
2) Pipette in positions B1/2 **50µl** each **Standard A (0 ng/ml)**,
   pipette in positions C1/2 **50µl** each **Standard B (0.1 ng/ml)**,
   pipette in positions D1/2 **50µl** each **Standard C (0.5ng/ml)**,
   pipette in positions E1/2 **50µl** each **Standard D (1 ng/ml)**,
   pipette in positions F1/2 **50µl** each **Standard E (2 ng/ml)**,
   pipette in positions G1/2 **50µl** each **Standard F (4 ng/ml)**,
   pipette in positions H1/2 **50µl** each **Standard G (8 ng/ml)**,

   To control the correct accomplishment, **50 µl** of the **1:16** (or in respective dilution rate of the sample) in Dilution Buffer **VP** diluted **Control Sera KS1** and **KS2** can be pipetted in positions A3/4 and B3/4.
   Pipette **50 µl each** of the **diluted samples** (generally 1:16 diluted in Dilution Buffer **VP,** please mix the dilutions immediately after sample addition and use within 60 minutes) in the rest of the wells, according to requirements.
3) Cover the wells with the sealing tape and incubate the plate for **1 hour** at **room temperature**
4) After incubation aspirate the contents of the wells and wash the wells **3 times** with **250 µl Washing Buffer WP**.
5) Following the last washing step, pipette **100 µl Enzyme Conjugate EK** in each well, **<u>except A1/A2</u>**.
6) Cover the wells with the sealing tape and incubate **30 min** at **room temperature**
7) After incubation wash the wells 3 times with **Washing Buffer WP** as described in step 4)
8) Pipette **100 µl of the TMB-Substrate solution S** in each well, **<u>also in A1/A2</u>**.
9) Incubate the plate for **15 Minutes in the dark at** room **temperature**.
10) After incubation pipette **100 µl Stop Solution SL** in each well **<u>also in A1/A2</u>**.
11) 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 G should be greater than 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 native hIGFBP-1:

Standard	**A**	**B**	**C**	**D**	**E**	**F**	**G**
ng/ml	0	0.1	0.5	1	2	4	8

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 absorbances 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 **concentration in ng/ml** of the samples can be calculated **by multiplication** with the respective dilution factor.

EXPECTATION VALUES

Concentrations of IGFBP-1 in human sera of 69 healthy adult donors were determined with the ELISA RMEE01R. Slight gender dependent differences were found, the concentrations of all samples varied from minimal 0.23 ng/ml to maximal 17.94 ng/ml (see table 5).

Table 5: Expectation values in sera of healthy adults (measured values in ng/ml)

Gender	**No. of Samples**	**Average value**	**Median**	**Min. – Max.:**
female	33	4.79	4.24	0.23 – 16.07
male	36	5.22	2.71	0.42 – 17.94
total	69	5.01	2,77	0.23 – 17.94

SUMMARY – IGFBP-1 ELISA RMEE01R

Reconstitution / Dilution of Reagents		
Standards A-G	Reconstitution in 500 µl **Dilution Buffer VP**	
Control Sera KS1 and KS2	Reconstitution in 250 µl **Dilution Buffer VP**	
Wash Buffer WP	dilute in **A. dest.** (eg. total volume of 50 ml in a graduated flask and fill up to 1000 ml)	**1:20**
Dilute Sample and **Control Sera KS1 and KS2** 1:16 with **Dilution Buffer DB**		
Before beginning the test procedure bring all reagents to room temperature.		

Assay Procedure for Double Determinations:

Pipette	**Reagent**	**Position**
IMPORTANT: Leave the position A1 / A2 empty until addition of Substrate		
50 µl	Antibody Conjugate **AK**	**In all wells except A1 / A2**
50 µl	Standard **A** (**0 ng/ml**)	B1 and B2
50 µl	Standard **B** (**0.1 ng/ml**)	C1 and C2
50 µl	Standard **C** (**0.5 ng/ml**)	D1 and D2
50 µl	Standard **D** (**1 ng/ml**)	E1 and E2
50 µl	Standard **E** (**2 ng/ml**)	F1 and F2
50 µl	Standard **F** (**4 ng/ml**)	G1 and G2
50 µl	Standard **G** (**8 ng/ml**)	H1 and H2
50 µl	1:16 diluted Control Serum **KS1**	A3 and A4
50 µl	1:16 diluted Control Serum **KS2**	B3 and B4
50 µl	1:16 diluted Samples	following wells
Cover the wells with the sealing tape.		

Incubation: 1 h at RT, without shaking

3x 250 µl	Aspirate the contents of the wells and wash **3x** with **250 µl Wash Buffer WP**	each well
100 µl	Enzyme Conjugate **EK**	each well, except A1/A2

Incubation: 30 min at RT, without shaking

3x 250 µl	Aspirate the contents of the wells and wash **3x** with **250 µl Wash Buffer WP**	each well
100 µl	Substrate **S**	each well

Incubation: 15 min in the dark **RT**

100 µl	Stop Solution **SL**	each well
Measure the absorbance within **30 min** at **450 nm** with **≥ 590 nm** as reference wavelength.		

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