Mouse/Rat IGFBP-2 ELISA

(Mouse and Rat Insulin-Like Growth-Factor Binding Protein-2)

Cat. No.: RMEE08R

TECHNICAL FEATURES

- Highly specific and sensitive assay for quantitative detection of IGFBP-2 in mouse and rat serum
- Recombinant mouse IGFBP-2 as standard
- Uses antibodies against complete mouse and rat IGFBP-2
- No sample extraction is required
- Detection limit: 0.01 ng/ml

INTRODUCTION

Insulin-like growth factors (IGFs) regulate the proliferation, differentation, apoptosis, cell adhesion and metabolism in various tissues and cell types. The IGF-1, which is produced mainly in liver under the influence of Growth Hormone (GH), regulates as hormone the linear growth of the bones and the process of sexual maturity, while IGF-II is mainly a growth factor of foetal tissue (11-13). The biological actions of IGF over the IGF-Type-1 receptor are modulated variably through the IGF binding proteins (IGFBP-1 to-6) (14). IGFBP-2 is, after IGFBP-3, the second most frequent IGFBP in the human blood. IGFs, especially tumor typical pro-IGF-forms and hormones regulate the expression of IGFBP-2, GH effect is thereby inhibiting. At cellular level IGFBP-2 seems to stimulate the proliferation and dissemination of solid tumors via an IGF-independent mechanism (15,16).

PHYSIOLOGICAL MEANING

IGFBP-2 is an unglycosylated polypetide of 31.3 kDa, which forms binary IGF-complexes and shows no circadian rhythm in the circulation.

The serum concentration of IGFBP-2 increases in fasting, after major surgery and after trauma, but the increasing of the concentration is most intensive in malignant diseases. The correlation of the IGFBP-2 level to the degree of progression is a striking feature in various tumor types as is the normalization of the IGFBP-serum levels after remission (5-10). During the GH-therapy, e.g. in short stature and in GH-abuse (doping) the IGFBP-2 level decreases. In Trisomy 18 IGFBP-2 in maternal serum is decreased and IGFBP-1 is increased; therefore the ratio IGFBP-2 /IGFBP-1 is a marker for this chromosome abnormality (17).

Transgenic organisms are a good opportunity to investigate the function of genes or proteins. The mouse or rat model is a well-suited system for investigation of the relevance of IGFBP-2 in physiological and pathological processes. Over expression of the IGFBP-2 gene in mice results in a weight reduction of 30% in spleen and moderately reduced weight in other organs (18). Effects of IGFBP-2 on the organism can be compensated through the modified expression of other IGF-Binding proteins.

Especially in tumor biology the mouse and rat systems enable investigation of the systemic relevance of IGFBP-2. IGFBP-2 influences tumor cells as it induces catalase activity in adrenocortical cells (19). Furthermore IGFBP-2 interacts with tumor cells via its RGD-amino acid sequence and seems to stimulate cell invasion of glioma cells (20).

INTENDED USE

This IGFBP-2 Enzyme Immunoassay-Kit is suited for quantitative determination of IGFBP-2 in mouse and rat serum for scientific purposes.

METHODOLOGY

Assay Characteristics and Validation

The ELISA for IGFBP-2 utilizes two different specific high affinity polyclonal antibodies for this protein. The ELISA recognizes quantitatively mouse and rat IGFBP-2 and is unaffected by an excess of IGF-I or IGF-II levels. Related molecules such as IGFBP-3 show no cross-reactions in the assay.

The standards are prepared of recombinant mouse-IGFBP-2 in the range of 0.03125 to 2 ng/ml.

The theoretical sensitivity of the assay is approx. 0.01 ng/ml (2 x SD of zero standard). Intra-assay and inter-assay variation coefficients were found both < 10%. Exemplary determinations are shown in the tables 1 and 2.

Table 1 : Inter-Assay-Variation

Sample 1	24.9 ng/ml	n= 6	CV = 6.4 %
Sample 2	105.7 ng/ml	n= 6	CV = 2.4 %
Sample 3	171.0 ng/ml	n= 8	CV = 5.3 %

Table 2: Intra-Assay-Variation

Sample	67.87 ng/ml	n = 9	CV = 4.6 %

Dilution of samples has been found over a wide range with very good linearity (see table 3).

Table 3: Linearity of the sample dilution:

Dilution	Mouse Serum **(ng/ml)**	**Rat Serum (ng/ml)**
1:20	>max.	23.06
1:40	>max.	23.81
1:80	109.08	24.10
1:160	118.30	25.73
1:320	125.42	27.73
1:640	127.48	32.14

Calibration

The assay has been calibrated against the recombinant Mouse-IGFBP-2 of R&D Systems Inc. (Minneapolis, USA; www.rndsystems.com).

Expectation Values

Several commercially available mouse and rat sera have been tested for their IGFBP-2 concentrations, following results were obtained :

	n	Median	min.	max.
Mouse Sera	5	**81.0 ng/ml**	59.7 ng/ml	105.7 ng/ml
Rat Sera	4	**24.2 ng/ml**	10.7 ng/ml	38.1 ng/ml

Significant variations of serum values depending on the individual animal or the respective strain or mutant are likely, prior verification is recommended.

Sample Preparation and Storage

Whole blood should be processed within two hours. Once separated the samples should be stored frozen until measurement. IGFBP-2 levels are influenced by improper handling or storage and do not remain stable over several days at elevated temperatures. Store undiluted samples frozen in a tightly closed plastic vial. **Repeated freezing and thawing of serum/plasma should be avoided,** it seems to have a measurable effect on IGFBP-2 levels. The high sensitivity of the assay allows measurement of IGFBP-2 in small sample volumes, which is limited by pipetting accuracy rather than the amount of IGFBP-2.

Serum samples should be diluted prior to measurement 1:20 – 1:500-fold with **Dilution Buffer VP,** depending on the expected values (see chapter Expectation Values). In general a dilution of 1:100 should be appropriate (the recommended minimal essential sample volume is: 10 µl serum).

Sample extraction is not required.

<u>Suggestion for dilution protocol (double determination):</u>

Mix 10 µl serum manually or with the aid of a dilutor with 990µl **Dilution Buffer VP** (1:100), or, more simple for larger series with 1000 µl Dilution Buffer VP (1:101).

If sample size is limiting, a minimum of 2.5 µl sample might be used alternatively, dilution in 250 µl VP yields a dilution of 1:101 (care should be taken to accuracy of pipetting such low volumes !).

Use 2 x 100 µl of this dilution in the assay.

MATERIALS

Materials 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 polyclonal anti-IGFBP-2 antibodies and packed in a laminate bag.
2)	CAL	**Standards A-G,** lyophilized: Contain recombinant mouse IGFBP-2: Standard values are between 31.25 - 2000 pg/ml (31.25; 62.5; 125; 250; 500; 1000; 2000 pg/ml) IGFBP-2 and have to be reconstituted with **1 ml** Dilution Buffer **VP** each.
3)	Control	**Control Serum KS,** lyophilized: Contains mouse serum and has to be reconstituted with **100 µl** Dilution Buffer **VP**. The exact concentration is given on the vial label.
4)	DILU	**Dilution Buffer VP**, 120 ml, ready for use.
5)	Ab	**Antibody Conjugate AK**, 120 µl, 100fold concentrated: Contains biotinylated anti-IGFBP-2 antibody and has to be diluted immediately before use **1:100** with Dilution Buffer **VP**.
6)	CONJ	**Enzyme Conjugate EK,** 120 µl, 100fold concentrated: Contains HRP-labelled Streptavidin and has to be diluted immediately before use **1:100** with Dilution Buffer **VP**.
7)	WASHBUF 20x	**Washing Buffer WP**, 50 ml, 20fold concentrated: Washing Buffer has to be diluted **1:20** with **A.dest.** before use.
8)	H_2SO_4	**TMB-substrate solution S,** 12 ml, ready for use.
9)	SUBST	**Stopping solution SL**, 0.4 N sulphuric acid, 12 ml, ready for use. *Caution, acid!*
10)		**Sealing tape** for covering of the Microtiter plate, 2 x

Materials not Provided

Precision pipettes and multichannel pipettes with disposable plastic tips

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

Vortex-mixer

Microtiter plate shaker (350 rpm)

Microtiter plate washer (recommended)

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

Polyethylene PE/Polypropylene PP tubes for dilution of samples

TECHNICAL RECOMMENDATIONS

Bring all reagents to room temperature (20 - 25°C) before use.

For the **reconstitution** of the lyophilised components **(Standards A - G** and **Control Serum KS)** the kit **Dilution Buffer VP** should 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.

Incubation at room temperature means: Incubation at 20 - 25°C.

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 become 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. 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 dynamicly 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.

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.

The shelf life of the components after opening is not affected, if used appropriately.

Store the unused microtiter wells airtight together with the desiccant at 2° to 8°C.

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

Reconstituted components (Standards A – G and Control Serum KS) should be stored at -20°C (or below) for up to 2 months. **Avoid repeated freeze-thaw cycles.**

In case you plan to perform multiple independent m/r-IGFBP-2 determinations over a longer period with one kit, you should aliquote the components prior to freezing into suitable smaller volumes.

This is strongly recommended. The diluted washing buffer is stable for 4 weeks at 2-8°C. It has to be at room temperature for usage!

PRECAUTIONS

For in-vitro 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 animal origin, therefore all components and specimens should be treated as potentially infectious.

Following components contain **0.01% 2-Methyl-4-isothiazolin-3-one Solution** as preservative: AK, EK, VP

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

Following components contain **0.01% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-isothiazol-3-**one as preservative: AK, EK, VP, WP

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. Store and Incubate in the dark.

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

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.

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.

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 Serum 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, **Antibody- Conjugate AK, Enzyme Conjugate** 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.

1. add **100 µl Dilution Buffer VP** in wells A1/2 (blank) and
2. pipette in positions B1/2 **100 µl Standard A,**
   pipette in positions C1/2 **100 µl Standard B,**
   pipette in positions D1/2 **100 µl Standard C,**
   pipette in positions E1/2 **100 µl Standard D,**
   pipette in positions F1/2 **100 µl Standard E,**
   pipette in positions G1/2 **100 µl Standard F**,
   pipette in positions H1/2 **100 µl Standard G.**
   To control correct accomplishment **100 µl** of the **(1:100)** diluted **Control Serum KS** can be pipetted in positions A3/4.
   Pipette **100 µl** of the **diluted sample** in the rest of the wells, according to requirements.
3. Cover the wells with sealing tape and incubate the plate for **1 hour** at **room temperature** (shake at 350 rpm).
4. After incubation aspirate the contents of the wells and wash the wells **5 times** with **300 µl** of **Washing Buffer WP** / well respectively.
5. Following the last washing step pipette **100 µl** of the of the **(1:100)** diluted **Antibody Conjugate AK** in each well, and incubate **1 hour** at **room temperature** (shake at 350 rpm).
6. After incubation wash the wells **5 times** with **Washing Buffer WP** as described above.
7. Following the last washing step pipette **100 µl** of the **(1:100)** diluted **Enzyme Conjugate EK** in each well, and incubate **30 min** at **room temperature** (shake at 350 rpm).
8. After incubation wash the wells **5 times** with **Washing Buffer WP** as described above.
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. Stop the reaction by adding **100 µl** of **Stopping Solution SL** to all wells.
12. Measure the absorbance within **30 minutes** at **450 nm** (**reference filter: ≥ 590 nm).**

EVALUATION OF RESULTS

Establishing the Standard Curve

For the evaluation of the assay it is preconditioned that the absorbance values of the blank should be below 0.25, these of standard G should be above 1.0.

Samples, which yield higher absorbance values than Standard G are beyond the standard curve, for reliable determinations these samples should be tested anew with a higher dilution.

Standards are provided in the following concentrations (use the concentration unit as preferred):

The standards provided contain the following concentrations of recombinant mIGFBP-2 :

Standard	**A**	**B**	**C**	**D**	**E**	**F**	**G**
pg/ml	**31.25**	**62.5**	**125**	**250**	**500**	**1000**	**2000**
ng/ml	0.03125	0.0625	0.125	0.25	0.5	1	2

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 absorbancies of all other values.
3) Plot the **Standard** concentrations **A-G** on the x-axis versus the mean value of the absorbancies of the **Standards** on the y-axis. By using the mean absorbancies of the samples herewith the sample concentrations can be received.
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-topoint alignment** in individual cases).
5) The m/rIGFBP-2 concentration of the samples can be calculated with the standard curve equation and by **multiplication** with the respective dilution factor.

REFERENCES

1. Elmlinger MW, Wimmer K, Biemer E, Blum WF, Ranke MB, Dannecker GE, (1996) Insulin-like growth factor binding protein 2 is differentially expressed in leukaemic T-and B-cell lines. Growth Regulation 6, 152-157
2. Hoeflich A, Nedbal S, Blum WF, Erhard M, Lahm H, Brem G, Kolb HJ, Wanke R, Wolf E. (2001) Growth inhibition in giant growth hormone transgenic mice by overexpression of insulin-like growth factor-binding protein-2. Endocrinology 142:1889-98
3. Ranke MB, Schweizer R, Elmlinger MW, Weber K, Binder G, Schwarze CP, Wollmann HA (2000) Significance of basal IGF-I, IGFBP-3 and IGFBP-2 measurements in the diagnostics of short stature in children. Hormone Research 54: 60-68
4. Ranke MB, Schweizer R, Elmlinger MW, Weber K, Binder G, Schwarze CP, Wollmann HA (2001). Relevance of IGF-I, IGFBP-3, and IGFBP-2 measurements during GH treatment of GH-deficient and non-GH-deficient children and adolescents. Hormone Research 55: 115-124
5. Crofton PM, Ahmed SF, Wade JC, Elmlinger MW, Ranke MB, Kelnar CJH, Wallace WHB (1999) Effects of a third intensification block of chemotherapy on bone and collagen turnover, insulin-like growth factor I, its binding proteins and short term growth in children with acute lymphoblastic leukemia. European Journal of Cancer 35: 960-967
6. Muller HL, Oh Y, Lehrnbecher T, Blum WF, Rosenfeld RG.(1994) Insulin-like growth factor-binding protein-2 concentrations in cerebrospinal fluid and serum of children with malignant solid tumors or acute leukemia. J Clin Endocrinol Metab. 79: 428-34
7. Martin W Elmlinger, Martin H Deininger, Burkhardt S Schuett, Richard Meyermann, Frank Duffner, Ernst H Grote, and Michael B Ranke (2001) In vivo expression of the insulin-like growth factor binding protein-2 in human gliomas increases with the tumor grade. Endocrinology 142: 1652-1658
8. Cohen P, Peehl DM, Stamey TA, Wilson KF, Clemmons DR, Rosenfeld RG (1993) Elevated levels of insulin-like growth factor-binding protein-2 in the serum of prostate cancer patients. J Clin Endocrinol Metab. 76: 1031-1035
9. Boulle N, Baudin E, Gicquel C, Logie A, Bertherat J, Penfornis A, Bertagna X, Luton JP, Schlumberger M, Le Bouc Y. (2001) Evaluation of plasma insulin-like growth factor binding protein-2 as a marker for adrenocortical tumors. Eur J Endocrinol. 144: 29-36
10. Ranke MB, Maier KP, Schweizer R, Stadler B, Schleicher s, Elmlinger MW, Flehmig B (2003), Pilot study of elevated levels of insulin-like growth factor-binding protein-2 as indicators of hepatocellular carcinoma. Horm Res 60:174-180
11. Rosenfeld RG, Roberts CT Jr. (eds.) (1999) The IGF system: Contemporary Endocrinology Series; Humana Press
12. Jones JI, Clemmons DR. (1995) Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev. 16: 3-34

13. Chard T (1994) Insulin-like growth factors and their binding proteins in normal and abnormal foetal growth. Growth Reg 4: 91-100

14. Ranke MB, Elmlinger MW (1997) Functional role of insulin-like growth factor binding proteins. Hormone Research 48 (suppl 4): 9-15

15. Elmlinger MW, Bell M, Schütt, BS, Langkamp M, Kutoh E, Ranke MB (2001) Transactivation of the IGFBP-2 promoter in human tumor cell lines. Molecular and Cellular Endocrinology 175: 211-218

16. Hoeflich A, Reisinger R, Lahm H, Kiess W, Blum WF, Kolb HJ, Weber MM, Wolf E. (2001) Insulin-like growth factor-binding protein 2 in tumorigenesis: protector or promoter? Cancer Res.15: 8601-8610

17. Miell JP, Langford KS, Jones JS, Noble P, Westwood M, White A, Nicolaides KH. (1997) The maternal insulin-like growth factor (IGF) and IGF-binding protein response to trisomic pregnancy during the first trimester: a possible diagnostic tool for trisomy 18 pregnancies. J Clin Endocrinol Metab.82: 287-292

18. Hoeflich A, Wu M, Mohan S, Foll J, Wanke R, Froehlich T, Arnold GJ, Lahm H, Kolb HJ, Wolf E. (1999) Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. Endocrinology 140(12):5488-5496.

19. Hoeflich A, Fettscher O, Preta G, Lahm H, Kolb HJ, Wolf E, Weber MM (2003) Increased activity of catalase in tumor cells overexpressing IGFBP-2. Horm Metab Res 35(11-12):816-821.

20. Song SW, Fuller GN, Khan A, Kong S, Shen W, Taylor E, Ramdas L, Lang FF, Zhang W. (2003) Iip45, an insulin-like growth factor binding protein 2 (IGFBP-2) binding protein, antagonizes IGFBP-2 stimulation of glioma cell invasion. Proc Natl Acad Sci USA 100(24):13970-13975.

Summary of the Assay

Reagent preparation:	Reconstitution:	Dilution:
Standards A-G	in **1 ml** Dilution Buffer **VP**	
Control Serum KS	in **100 µl** Dilution Buffer **VP**	**1:100 with Dilution Buffer VP**
Antibody Conjugate AK		**1:100** with Dilution Buffer **VP**
Enzyme Conjugate EK		**1:100** with Dilution Buffer **VP**
Washing Buffer WP		**1:20** with **Aqua. dest.** (e.g., add the complete contents of the flask **(50 ml)** into a graduated flask and fill with A.dest. to 1000 ml).
Sample dilution: e.g. 1:100 (e.g. Mix 10 µl Serum with 990 µl Dilution Buffer **VP**).		

Assay Procedure for double determination

	Reagents	Well positions
100 µl	Dilution Buffer **VP** (Blank)	A1/2
100 µl	Standard **A** (31.25 pg/ml)	B1/2
100 µl	Standard **B** (62.5 pg/ml)	C1/2
100 µl	Standard **C** (125 pg/ml)	D1/2
100 µl	Standard **D** (250 pg/ml)	E1/2
100 µl	Standard **E** (500 pg/ml)	F1/2
100 µl	Standard **F** (1000 pg/ml)	G1/2
100 µl	Standard **G** (2000 pg/ml)	H1/2
100 µl	Control Serum **KS**	A3/4
100 µl		following wells
Cover the wells with the sealing tape.		
Incubation: 1 h at RT, ≥350 rpm		
5x 300 µl	Aspirate the contents of the wells and **wash** 3x with **300 µl** Wash Buffer **WP**	each well
100 µl	1:100 diluted Antibody Conjugate **AK**	each well
Incubation: 1 h at RT, ≥350 rpm		
5x 300 µl	Aspirate the contents of the wells and **wash** 3x with **300 µl** Wash Buffer **WP**	each well
100 µl	1:100 diluted Enzyme Conjugate **EK**	each well
Incubation: 30 min at RT, 350 rpm		
5x 300 µl	Aspirate the contents of the wells and **wash** 3x with **300 µl** Wash Buffer **WP**	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 with ≥ 590 nm** as reference wavelength.		

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