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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-I, 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-I 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.

Suggestion for dilution protocol (double determination):

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) **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) **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 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) **Dilution Buffer VP**, 120 ml, ready for use.

5) **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) **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) **Washing Buffer WP**, 50 ml, 20fold concentrated: Washing Buffer has to be diluted **1:20** with **A.dest.** before use.

8) **TMB-substrate solution S,** 12 ml, ready for use.

9) **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

- Distilled or demineralized water for dilution of the **Washing Buffer WP**
- Micropipettes and multichannel pipettes with disposable plastic tips
- Vortex-mixer
- Device to aspirate the standards and the samples from the wells
- Plate washer and plate shaker (recommended)
- Microplate reader ("ELISA-Reader") with filter for 450/620 nm wavelengths
- Foil welding device for laminate bags (recommended)

TECHNICAL RECOMMENDATIONS

In conducting the assay, follow strictly the test protocol.

Reagents with different lot numbers should not be mixed.

The microtiter plate and all reagents are stable until the expiry date if stored in the dark at 2-8°C (s. label).

The kit **Dilution Buffer VP** should be used for the **reconstitution** of the lyophilized components **(Standards A - G** and **Control Serum KS)**. 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 **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 Serum KS**) 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 m/rIGFBP-2 determinations over a longer period with one kit, you should aliquot the components prior to freezing into suitable smaller volumes. This is strongly recommended.

The 1:20 diluted **Washing Buffer WP** is stable only limited. Please dilute only according to requirements.
This applies to the 1:100 diluted **Antibody Conjugate AK** and **Enzyme Conjugate EK** solutions too.

Before use, all kit components should be brought **to room temperature**. Room temperature incubation means: incubation at 20 - 25°C. Precipitates in buffers should in case be dissolved before use thorough 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 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, the 1:100 diluted **Antibody Conjugate AK** and the **Enzyme Conjugate EK** solutions as well as the succeeding **Substrate Solution S** solution 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**.

PRECAUTIONS

The kit should not be used beyond the expiration date on the kit label.

All reagents are for in vitro use only! In conducting the assay, follow strictly the test protocol. The acquisition, possession and use of the kit are subjects to the regulations of the national regulatory authorities.

Reagents with different lot numbers should not be mixed.

The Stop Solution provided is an acid solution. Avoid direct contact. Wear eye, hand, face and clothing protection when using this material.

The handling of potentially infectious material must comply with the following guidelines:

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.

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.

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.

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.

ASSAY PROCEDURE

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

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 into a disinfectant (risk of infection!) and wash the wells 3 times with **250 µl** of **Washing Buffer WP** / well respectively. **Washing Buffer WP** should incubate at least for **15 seconds**/well.

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 3 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 3 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: 620 nm).

EVALUATION OF RESULTS

Establishing the Standard Curve

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 (MA) value for the blank from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance (MA) 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 standard curve and sample concentrations should be done by using a computer programme, because the standard curve is in general best described by a non-linear regression or a higher-grade polynom or four parametric (4PL) curve fits.
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.

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		
3x 250 µl	Aspirate the contents of the wells and **wash** 3x with **250 µl** Wash Buffer **WP**	each well
100 µl	1:100 diluted Antibody Conjugate **AK**	each well
Incubation: 1 h at RT, ≥350 rpm		
3x 250 µl	Aspirate the contents of the wells and **wash** 3x with **250 µl** Wash Buffer **WP**	each well
100 µl	1:100 diluted Enzyme Conjugate **EK**	each well
Incubation: 30 min at RT, ≥350 rpm		
3x 250 µl	Aspirate the contents of the wells and **wash** 3x with **250 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 620 nm** as reference wavelength.		

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