Mouse/ Rat IGFBP-3 ELISA

Cat. No.: RMEE031R

TECHNICAL FEATURES + APPLICATIONS

- Quantitative determination of mouse/rat IGFBP-3 without sample pretreatment
- Inter-Assay variation of 9.06% and Intra-Assay variation of 4.63%
- Analytical sensitivity of 0.018 ng/mL (18 pg/mL; 1,8 pg/well)

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.

Been made e.g. also by the nourishing situation, different diets about the IGFBP-3 concentrations affects a regularization.

INTENDED USE

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

PERFORMANCE CHARACTERISTICS AND VALIDATION

The Mediagnost ELISA for mouse/rat IGFBP-3 (m/rIGFBP-3) E031 is a so-called Sandwich-Assay. It utilizes two specific and high affinity antibodies for this protein. The IGFBP3 in the sample binds to the immobilized first antibody on the microtiter plate. In the following step, the biotinylated and Streptavidin- Peroxidase conjugated second specific anti-mouse IGFBP-3-Antibody binds in turn to the immobilised mIGFBP-3. In the closing substrate reaction the turn of the colour will be high specific catalysed, quantitatively depending on the m/rIGFBP-3-level of the samples.
The standards of the ELISA E031 are **recombinant mouse IGFBP-3** in concentrations of **0.078, 0.156, 0.313, 0.625, 1.25, 2.50 and 5 ng/mL.**

Sensitivity
The **analytical sensitivity** of the ELISA E031 yields **0.018 ng/mL** (2 SD of zero standard in 21fold determination).

The **Inter**- and **Intra-Assay** variation coefficients were found less than **9,06 %** and **4.63 %.** Exemplary determinations are shown in table 1 and table 2.

Table 1: Inter-Assay-Variation (n=61 or 56)

	Mean Value	Standard Deviation	VC
Sample 1	484,3	38,50	8,00
Sample 2	343,6	31,15	9,06

Table 2: Intra-Assay-Variation (n=16)

	Mean Value	VC
Sample 1	123.8	4.63
Sample 2	395.0	1.99

Specificity

Serum of the cited species was diluted (1:301) and used as sample 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 IGFBP3: 0.03%

The recovery of rec. 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.

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

Dilution:	Sample 1 (recalculated, ng/ml)	Sample 2 (recalculated, ng/ml)
1:100	351.8	367.6
1:200	369.1	414.5
1:400	384.5	423.4
1:800	381.3	411.0
1:1600	379.2	421.9
1:3200	386.1	455.7
AV / 1SD / VC%	375.3 / 12.9 / 3.46	415.7 / 28.4 / 6.83

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

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

Mouse and Rat Serum-, EDTA- and Citrate- (in this case please note the usual dilution of the sample collection vial) Plasma samples are applicable. In Heparin-Plasma samples the levels were found approx. 15% decreased. Further, cell culture medium was found to be suitable.

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.

Storage of samples at -20°C min. 2 years.

The storage of samples over a period of 2 years at -20°C, showed no effect on the measured value.

Avoid repeated freeze-thaw cycles of samples (if required, please subaliquote) although m/rIGFBP-3 levels were found to be unaffected by three cycles in our experiments.

The high sensitivity of the assays allows m/rIGFBP-3 determinations in small sample volumes,

which is limited by pipetting accuracy rather than the amount of m/rIGFBP-3.
In most determinations (e.g. serum- or plasma samples and no extreme values expected) the dilution of **1:301 with Dilution Buffer VP is suitable**, the respective covered range would be 0.018 to 1505 ng/mL. Where required, depending on the expected IGFBP-3-values, the dilution with **Dilution Buffer VP** can be higher or lower.

Suggestion for dilution protocol:
Pipette 1.5 mL Dilution Buffer VP in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add 5 µL Serum- or Plasma (dilution 1:301) and mix each tube immediately. After mixing use 100 µL of this solution within 1 hour per determination in the assay.

REAGENT 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-mouse/rat IGFBP-3 Antibody, packed in a laminate bag.
2)	CAL	**Standards A-G**, lyophilised, contain recombinant mouse IGFBP-3. Standard values are between 0.078 – 5 ng/mL (0.078, 0.156, 0.313, 0.625, 1.25 2.5 and 5 ng/mL) mIGFBP-3, Standards are reconstituted with 750 µL Dilution Buffer VP each. Use 100 µL pro well in the assay.
3)	BUF X	**Dilution Buffer VP, 125 mL**, ready for use.
4)	Controls	**Control Sera KS1 and KS2, 100 µL**, lyophilised. **KS1** contains mouse serum and **KS2** rat serum. Please reconstitute in **100 µL Dilution Buffer VP**. The m/rIGFBP-3 target values and the respective ranges are given on the label of the vial. The dilution should be according to the dilution of the respective samples.
5)	Ab	**Antibody Conjugate AK, 12 mL**, contains biotinylated anti-mouse IGFBP-3 antibody, ready for use. Pipette 100 µL per well.
6)	CONJ	**Enzyme Conjugate EK, 12 mL**, contains HRP (Horseradish-Peroxidase)- labelled Streptavidin, ready for use. Pipette 100 µL per well.
7)	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 4 weeks stable at 2-8°C. Please dilute only according to daily requirements.
8)	SUBST	**Substrate S, 12 mL**, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H_2O_2 Tetramethylbencidine.
9)	H_2SO_4	**Stopping Solution (SL),** 12 ml, ready for use, 0.2 M sulphuric acid, Caution acid!
10)		Sealing tape for covering of the microtiter plate, 2 x, adhesive.

MATERIALS REQUIRED BUT NOT PROVIDED

Precision pipettes and multichannel pipettes with disposable plastic tips
Distilled or deionized water for dilution of the Washing Buffer (WP), 950 mL Graduated cylinder for diluting 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
Polyethylen PE/Polypropylen PP tubes for dilution of samples

REAGENT PREPARATION

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 unopened until the expiry date, if stored in the dark at 2°-8°C (see label).
The shelf life of the components **after initial opening is** warranted for **4 weeks**, if stored appropriately.
Before use, all kit components should be brought to room temperature at 20 - 25°C. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming.
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 dynamically 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.

Standards and Controls

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.

The reconstituted standards **A-G** and controls **KS1 a**nd **KS2** can be stored for **4 weeks at –20°C.**

Repeated freeze/thaw cycles have to be avoided (3 cycles in our experiments didn´t have an influence on the assay).

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 **Washing Buffer**

The 1:20 diluted **Washing Buffer WP** is 4 weeks stable at 2-8°C. Please dilute only according to daily requirements.

Microtiter plate

After initial opening, store the unused strips and **microtiter wells** airtight together with the desiccant at 2-8°C in the clip-lock bag. Use it in the provided frame.

Substrate Solution

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

WARNINGS AND 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. The Mediagnost GmbH is not liable for any loss or harm caused by non-observance of the instructions, as far as no law withstands.

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.

Disposal of containers and unused contents should be done in accordance with federal and local regulatory requirements.

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

Do not use expired reagents.

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.

Caution: This kit contains material of human and/or animal origin, therefore all components and specimens should be treated as potentially infectious.

Animal sera of Mouse and/or Rat origin are contained in controls KS1 and KS2.

Stop solution contains 0.2 M Sulfuric 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.

2-Methyl-4-Isothiazolin-3-one

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

< 0,01% 2-Methyl-4-isothiazolin-3-one Solution

R34 Causes burns

R43 Sensibilisation through skin contact possible

S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice

S36/37 Wear suitable protective clothing and gloves

S45 In case of accident or if you feel unwell seek medical advice

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

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

< 0,01% (w/w) 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-Isothiazol-3-one Solution

R36/38 Irritating to eyes and skin

R43 Sensibilisation through skin contact possible

S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice

S28.1 After contact with skin, wash immediately with plenty of water

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

R20/21/R22 Harmful by inhalation, in contact with skin and if swallowed

R36/37/38 Irritating to eyes, respiratory system and skin

S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice

S28.1 After contact with skin, wash immediately with plenty of water

S36/37 Wear suitable protective clothing and gloves

General first aid procedures:

Skin contact: Wash affected area thoroughly with water. Discard contaminated cloths and shoes.

Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing spread the eyelids.

Ingestion: If swallowed, wash out mouth thoroughly with water. Immediately see a physician.

Do not eat, drink or smoke in these areas.

Never pipette the materials with the mouth.

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

ASSAY PROCEDURE

NOTES: All determinations (Standards, Control 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** and **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.

1) Add **100µL Dilution Buffer VP** in positions A1/2.

2) Pipette in positions B1/2 **100 µL** each **Standard A (0.078 ng/mL)**,
pipette in positions C1/2 **100 µL** each **Standard B (0.156 ng/mL)**,
pipette in positions D1/2 **100 µL** each **Standard C (0.313 ng/mL)**,
pipette in positions E1/2 **100 µL** each **Standard D (0.625 ng/mL)**,
pipette in positions F1/2 **100 µL** each **Standard E (1.25 ng/mL)**,
pipette in positions G1/2 **100 µL** each **Standard F (2.5 ng/mL)**,
pipette in positions H1/2 **100 µL** each **Standard G (5 ng/mL)**.

To control the correct accomplishment **100 µL** of the 1:301 (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 **100 µL each** of the **diluted sample** (generally 1:301 diluted in Dilution Buffer **VP**) in the rest of the wells, according to requirements. Please mix the dilutions immediately after sample addition and use within 60 minutes.

3) Cover the wells with the 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 Washing Buffer WP**.

5) Following the last washing step, pipette **100 µL** of the **Antibody Conjugate AK.** Cover the wells with the sealing tape 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 in step 4)

7) Following the last washing step, pipette **100 µL** of the **Enzyme Conjugate EK.** Cover the wells with the sealing tape and incubate **15 min** at **room temperature** (shake at 350 rpm).

8) After incubation aspirate the contents of the wells and wash the wells **5 times** with **300 µL Washing Buffer WP**.

9) Pipette **100 µL of the TMB-Substrate solution S** in each well.

10)Incubate the plate for **15 Minutes in the dark at** room **temperature**.

11)After incubation pipette **100 µL Stop Solution SL** in each well.

12)Measure the absorbance **within 30 minutes at 450 nm** (Reference filter **≥590 nm**).

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	0.078	0.156	0.313	0.625	1.25	2.5	5

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 3 ng/mL, Sample was 1:301 diluted: 3 x 301= 903 ng/mL, or 0.903 µg/mL or 0.903 mg/L, according the requested unit).

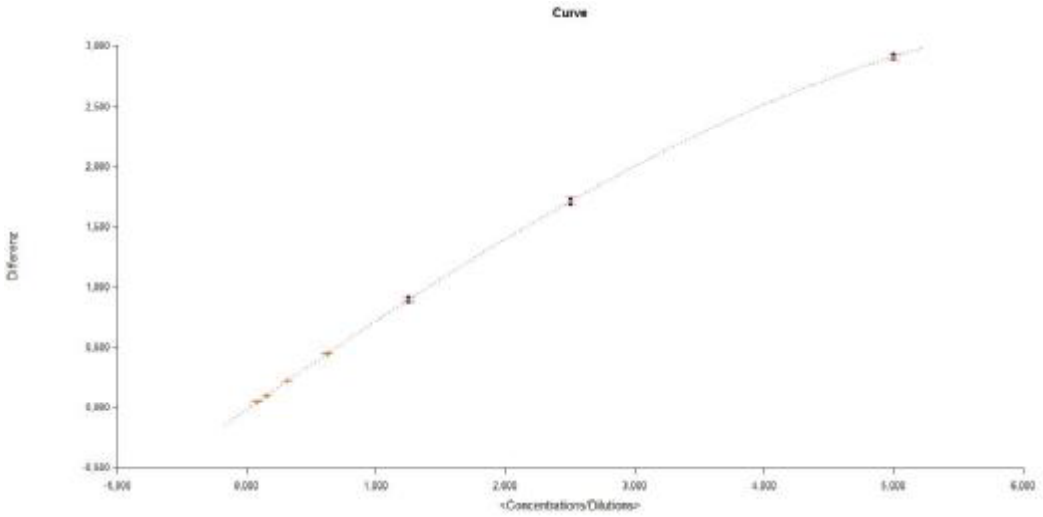

Fig. 1: Exemplary Standard Curve with a polynomial 3rd degree as curve fit.

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 of your sample 1.0115

Measured extinction of the blank 0.1045

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:

$0.91 = -0.00339 \times X^3 + -0.0166 \times X^2 + 0.753 \times X - 0.0166$

$1.265 = X$

Multiplication by dilution factor (1:301) gives the IGFBP-3 concentration of the sample with 380.7 ng/mL

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SUMMARY – MOUSE/RAT IGFBP-3 ELISA RMEE031R

Reconstitution/ Dilution of Reagents		
Standards A-G	Reconstitution in **Dilution Buffer VP**	**750 µl each**
Control Sera KS1 and KS2	Reconstitution in **Dilution Buffer VP**	**100 µl**
Washing Buffer WP	dilute in **A. dest.** (e.g. add the complete contents of the flask 50 ml into a graduated flask and fill with A.dest. to 1000 ml)	**1:20**
Sample Dilution + Control Serum KS1 and KS2: 1:301 in Dilution Buffer VP, mix directly and use within max. 60 min. Use 100 µL per determination.		
Before assay procedure bring all **reagents** to **room temperature**		

Proposal of Assay Procedure for Double Determination:

Pipette	Reagents	Well Positions
100 µl	Dilutionbuffer **VP** as Blank	A1 and A2
100 µl	Standard **A** (**0,078 ng/ml**)	B1 and B2
100 µl	Standard **B** (**0,156 ng/ml**)	C1 and C2
100 µl	Standard **C** (**0,313 ng/ml**)	D1 and D2
100 µl	Standard **D** (**0,625 ng/ml**)	E1 and E2
100 µl	Standard **E** (**1,25 ng/ml**)	F1 and F2
100 µl	Standard **F** (**2,5 ng/ml**)	G1 and G2
100 µl	Standard **G** (**5 ng/ml**)	H1 und H2
100 µl	Control Serum **KS1**	A3 und A4
100 µl	Control Serum **KS2**	B3 und B4
100 µl	**Sample dilution**	Pipette sample in the rest of the wells according to requirements
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 **5x** with **300 µL** each **WP/well**	each well
100 µl	Antibody Conjugate **AK**	each well

Incubation: 1 h at RT, 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

Incubation: 15 min at RT, 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

Incubation: 15 min at RT, 350 rpm

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

STD A-G	A-G	Rec in 750 µl BUF VP	100 µl
Control	KS1 and KS2	Rec in 100 µl BUF VP	100 µl
WASHBUF 20x	WP		1:20 DILU A. dest.
SPE + Control 1:301 DILU BUF VP ↔ ⏲ max. 1h			100 µl
°C 20-25 °C			

100 µl	BUF VP	A1/2
100 µl	STD A (0.078 ng/ml)	B1/2
100 µl	STD B (0.156 ng/ml)	C1/2
100 µl	STD C (0.313 ng/ml)	D1/2
100 µl	STD D (0.625 ng/ml)	E1/2
100 µl	STD E (1.25 ng/ml)	F1/2
100 µl	STD F (2.5 ng/ml)	G1/2
100 µl	STD G (5 ng/ml)	H1/2
100 µl	CONTROL KS1 1:301 DILU BUF VP	A3/4
100 µl	CONTROL KS2 1:301 DILU BUF VP	B3/4
100 µl	SPE 1:301 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

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