Human Growth Hormone Sensitive ELISA

(Growth Hormone)

Cat. No.: RMEE022

IVD CE

CLINICAL RELEVANCE

Determination of human Growth Hormone (hGH, Somatropin) is done for diagnostic of Growth Hormone deficiency or Growth Hormone excess (arcomegaly). During medicinal and/or after surgical therapy of arcomegaly Growth Hormone (and IGF-I) measurement is used for therapy control.

TECHNICAL PROPORTIES AND APPLICATIONS

hGH ELISA RMEE022

- is well suited for determination of hGH in **serum** and **plasma** (native samples, hGH profiling, samples of stimulation and suppression tests)

- due to its high **sensitivity** (0,0016 ng/ml ≅ 1,6 pg/ml) it is also very well suited to measure hGH in **cell culture supernatants** and other **non-serum samples (e.g. hGH urine – excretion test)**

- is calibrated against **the reference assay** of the KIGS/IGLU study, in house-RIA of University Children Hospital Tübingen (see literature 1)

- is highly **specific** and therefore allows measurement of hGH during simultaneous application of hGH and hGH-analoga e.g. Pegvisomant (e.g. Somavert® of the Pfizer Inc.)

- is fast: total incubation time is only 3.5 hours

- is calibrated against recombinant hGH (International Standard, **NIBSC 98/574**; see literature 6)

- Single Standards of **50, 150, 300, 600 bzw. 1000 pg/ml** are supplied within our kit, control serum consisting of human serum is also provided

- applies high affine polyclonal antibodies against 22 kDa recombinant hGH

INTRODUCTION

The endocrine system of human Growth Hormone (hGH), also named Somatropin, is characterized by an extreme complexity. hGH is the product of the GH-1 gene located on chromosome 17 and expressed in pituitary cells. 80% of the gene expression results in a non-glycolsylated 22 kDa protein consisting of 191 amino acids. The other 20% of gene expression results in a variant form of 20 kDa by alternative splicing. Additionally, several more smaller variants can be found in circulation as well as translational modificated proteins and different degrees of protein aggregation. Further on, bioactivity of Growth Hormone is regulated by a specific binding protein (GHBP) formed by the extra cellular part of the cellular transmembran GH-receptor. These modifications allow a tight control of the half-life period hGH and of its bioactivity. GH is species specific.

Not only synthesis and posttranslational modification but also secretion of hGH is tightly regulated. Spontaneous pulsatile secretion takes place with a single pulse every three hours and a maximal secretion during night's sleep. Several different attractions as physiologic stress or hypoglycaemia and amino acids result in additional hGH secretion, induced by the hypothalamic hormones Somatostatin and GH-Releasing Hormone (GHRH). Age, sexual steroids, nutritional status, illness and emotions influence the amount of secreted hGH. Because of the multitude of influencing factors the normal quantitative secretion is not known.
Physiological functions are partially exerted by Insulin-like Growth Factors (IGFs). In children and adolescent the hGH system is the main regulator of growth. If the hGH system fails totally, human growth will end at 120 cm. Beside regulation of growth hGH exerts an anabolic effect on muscle and connective tissue as wells as on bone and different other organs (heart, intestine). Further hGH was proved to have a lipolytic effect.

Growth Hormone pathology is characterized by extreme high or extreme low hGH secretion. During childhood it is the Growth Hormone deficiency congenital or acquired, which leads to microsomia. For **diagnosis of Growth Hormone deficiency an hGH stimulation test has to be done or the spontaneous excretion must be investigated**. The therapy consists of substitution of endogenous Growth Hormone by recombinant hGH resulting in normalization of growth.

In adulthood hGH deficiency is mostly caused by pituitary adenoma (and their surgical excision). hGH deficiency shows typical disease pattern, equivalent to advanced aging (adipositas, muscle dystrophy, arteriosclerosis, osteoporosis, adynamia). Substitutional therapy is a well-known, approved and efficient therapy of severe Growth Hormone deficiency in adulthood. Therapeutical success is directly as well as indirectly proved by measurement of IGF in serum.

Excessive hGH secretion, mostly causes by pituitary adenoma, results in childhood in gigantism, in adulthood in acromegalie, leading to enlarged extremities, diabetes, heart insufficiency and tumor growth. Surgical excision of the adenoma is the therapy of choice. If tumor excision is not possible or incomplete, a medicinal therapy with somatostatin preparation will be conducted, resulting in inhibition of hGH production. Alternatively hGH analoga (e.g. Pegvisomat) are used to block the hGH receptor and thereby inhibit action of endogenous hGH. Determination of human Growth Hormone (hGH, Somatropin) is done for diagnostic of Growth Hormone deficiency or Growth Hormone excess (arcomegaly). During medicinal and/or after surgical therapy of arcomegaly Growth Hormone (and IGF-I) measurement is used for therapy control.

PRINCIPLE

The hGH SENSITIVE ELISA E022 is a so-called sandwich-assay. It utilizes a specific, high affinity polyclonal rabbit antiserum coated on the wells of a microtiter plate. The hGH in the samples binds quantitatively to the immobilized antiserum. In the following step, the biotinylated antiserum in turn binds hGH. After washing, a streptavidin-peroxidase-enzyme conjugate will be added, which will bind highly specific to the biotin of the antiserum and will catalyze the substrate to change the color quantitatively depending on the hGH level of the sample.

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 Serum 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 Sulfuric Acid (H_2SO_4)

R36/38 rritating 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.Some reagents contain <0.1% Kathon CG and <0.1% Proclin as preservatives. In case of contact with eyes or skin, flush immediately with water.

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.

REAGENTS PROVIDED

1)	MTP	**Microtiter plate**, ready for use: Microtiter plate with **96 wells**, divided into 12 strips with 8 **break-apart wells** coated with human Growth Hormone
2)	CAL	**Standards (A-E)**, lyophilized: Contain recombinant hGH (NIBSC 98/574). Standard values are between **0.05 - 1 ng/m**l (50, 150, 300, 600 and 1000 pg/ml) hGH. Each vial must be reconstituted with **750 µL of Dilution Buffer (VP)**.
3)	**DILU X**	**Dilution Buffer (VP)**, 120 ml, ready for use, please use for the reconstitution of Standards A – E and of Control Serum (KS) as well as for the sample dilution
4)	Control	**Control Serum (KS)**, lyophilized, contains human serum and must be reconstituted with **100 µL Dilution Buffer VP**. The hGH target value concentration and the respective range are given on the vial label. The dilution of the KS should be according to the dilution of the respected samples
5)	Ab	**Antibody Conjugate (AK),** 12 ml, ready-made solution, contains rabbit biotinylated anti-hGH antibody**..** Use 100 µl per Well in the Assay.
6)	CONJ	**Enzyme Conjugate (EK),** 12 ml, ready-made solution, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin. Use 100 µl per Well in the Assay.
7)	WASHBUF 20x	**Washing Buffer (WP),** 50 ml, 20 X concentrated solution. Washing buffer has to diluted 1:20 with A.dest. or demineralised water before use (e.g. add the complete contents of the flask 50 ml into graduated flask and fill with A.dest to 1000 ml). Attention: After dilution, the Washing Buffer is only limited stable, please dilute only according to 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 (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

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

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.

Washing Buffer (WP) has to be diluted 1:20 with distilled or demineralised water before use (e.g. add the complete contents of the flask (50 ml) into a graduated flask and fill up with A.dest. to 1000 ml). Attention: After dilution the Washing Buffer is only limited stable, dilute only according to requirements.

The **Dilution Buffer (VP)** must be used for the reconstitution of the lyophilized components (**Standards A – E and Control Serum KS)**. Each bottle of Standards must be reconstituted with 750 µl Dilution Buffer. Control Serum (KS) must be reconstituted with 100µL of Dilution Buffer. 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.

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 (Standards (A – E) 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 hGH determinations over a longer period with one kit, you should aliquot the components prior to freezing into suitable smaller volumes. This is strongly recommended.

SPECIMEN COLLECTION, PREPARATION, AND STORAGE

Serum as well as plasma samples are suitable (significant deviation of hGH levels in corresponding Serum, Heparin-, or EDTA-Plasma samples were not found). Common cell culture medium was found to be suitable. An external sample preparation prior to assay is not required (see below).Samples should be handled as recommended in general: collected and refrigerated as fast as possible. In case there will be a longer period (>24 hours) between the sample withdrawal and determination, store the undiluted samples frozen at –20°C or below in tightly closable plastic tubes. Avoid on principal repeated freeze-thaw cycles of serum/plasma (if required, please sub-aliquot) although hGH levels were found to be unaffected by a few cycles, (5x) in our

experiments. The high sensitivity of the assay allows hGH measurement of hGH in small sample volumes. In most determinations (serum or plasma samples, and no extreme values expected) a dilution from 1:10 to 1:50 with Dilution Buffer (VP) should be suitable. According to expected hGH levels, the dilution with Buffer can be higher or lower. In general, a dilution of 1:26 for serum- or plasma samples is appropriate. The hGH concentrations may be completely different in body fluids of human origin other than serum or cell culture supernatants.

Suggestion for dilution protocol:

Pipette 250 µL Dilution Buffer VP in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series); add 10 µL serum- or plasma samples (dilution 1:26). After mixing use 2 x 100 µL of this dilution in the assay.

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 and the Enzyme Conjugate as well as the succeeding Substrate Solution should be added to the plate in the same order and in the same time interval as the samples. Stop Solution should be added to the plate in the same order as the Substrate Solution.

1) Add **100µL Dilution Buffer VP** in wells A1/A2 (blank).
2) Pipette in positions **B1/2 100 µl of Standard A (0.05 ng/ml)**

   Pipette in positions **C1/2 100 µl Standard B (0.15 ng/ml)**,

   Pipette in positions **D1/2 100 µl Standard C (0.3 ng/ml)**,

   Pipette in positions **E1/2 100 µl Standard D (0.6 ng/ml)**,

   Pipette in positions **F1/2 100 µl Standard E (1 ng/ml)**.

   For control purpose pipette 100 µl of the 1:26 (or respective dilution as the sample)

   diluted Control Serum in positions G1/2.

   Pipette **100 µl of each of the diluted samples** (e.g. diluted 1:26 or other) into the rest of the wells.
3) Cover the wells with sealing tape and incubate the plate for **2 hours 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 / well. Aspirate wells after each washing. Following the last washing step, bang the plate inverted onto a paper towel to remove residual liquid.
5) Pipette **100 µl of the Antibody Conjugate** into each well.

6) Cover the wells with sealing tape and incubate the plate for **0.5 hour** at room temperature (shake at 350 rpm).

7) Subsequently –**without a washing step!** - pipette **100 µl** of the **Enzyme-Conjugate** in each well and incubate additional **30 minutes without shaking**

8) After incubation wash the wells 5 times with Washing Buffer as described in step 4.

9) Pipette **100 µl of the TMB Substrate** Solution in each well.

10) Incubate the plate for **15 minutes in the dark at room temperature (20 - 25°C)**.

11) Stop the reaction by adding **100 µl of Stopping Solution**.

12) Measure the colour reaction within 30 minutes at 450 nm (reference filter ≥ 590 nm).

QUALITY CONTROL

Good laboratory practice requires that controls be run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards/laws. All standards and kit controls must be found within the acceptable ranges as stated on the QC Certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

CALCULATION OF RESULTS

Establishing the Standard Curve

The 2nd International Standard for hGH, NIBSC Code 98/574, was used as standard material(6). This was defined in an international study in the year 2001 with 3 International units per mg Protein (3 IU/mg). The exclusive application of this standard material is recommended in line with the current standardisation efforts for hGH Immunoassays. (7,8)

For the evaluation of the assay it is required that the absorbance values of the blank < 0.2, and of standard E ≥ 1.00. Samples, which yield higher absorbance values than Standard E should be re-tested with a higher dilution.

Standard	**A**	**B**	**C**	**D**	**E**
ng/ml	0.05	0.15	0.30	0.60	1.0
pg/ml	50	150	300	600	1000
µIU/ml	0.15	0.45	0.9	1.8	3.0

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 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 on semi-log paper (lin-log).
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. **Non-linear regression**, a higher-grade polynomial or four parametric logistic (4-PL) lin-log curve fit are suitable for the evaluation. A good fit is provided with cubic spline, 4 Parameter Logisitcs or Logit-Log.
5) The hGH concentration in ng/ml (or pg/ml, or µIU/ml, according the chosen unit for the standards) of the samples can be calculated by multiplication with the respective dilution factor.

INTERPRETATION OF RESULTS

The cut-off value is determined as a maximal peak of growth hormone secretion in at least 2 independent stimulation assays (e.g. insulin or arginine stimulation). Using WHO standard 98/574, which is equivalent to standard material used in this assay, a secretion peak of less than **8 ng/ml indicates** a possible growth hormone deficiency. But as growth hormone secretion is continous between normal and pathological any cut-off is only a non-binding benchmark. **Further diagnostic measurements** should be carried out to approve the results of this test. And every laboratory should establish its own cut-off values corresponding to the relevant group of patients.

EXPECTED NORMAL VALUES

As growth hormone is secreted pulsatile mainly enduring the night sleep valid normal values can hardly be determined. Standard procedures are arginin or insulin stimulation tests, after injection of stimulating substance growth hormone concentration is measured over a period of time. We investigated hGH serum concentration of 104 healthy blood donors in the age of 18-69 years without any stimulation.

	female	**male**
number	54	50
median [ng/ml]	0.81	0.28
minimal concentration [ng/ml]	0.19	0.15
maximal concentration [ng/ml]	10.15	4.34

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests. Furthermore, we recommend that each laboratory determine its own range for the population tested.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity of the hGH SENSITIVE ELISA E022 yields 0.0016 ng/ml (equal to 1.6 pg/ml, equal to 0.16 pg per well; 2x SD of zero standards in 16-fold determination).

Specificity

The only human protein with significant sequence similarities to growth hormone is prolactin. Testing a 200ng/ml prolactin solution in this assay, no cross reactivity was detected.

Intra-Assay-Variation

	Number of determinations	Mean value (ng/ml)	Standard deviation (ng/ml)	CV (%)
Sample 1	16	0.45	0.02	3.65
Sample 2	22	5.94	0.13	2.16

Inter-Assay-Variation

	Number of determinations	Mean value (ng/ml)	Standard deviation (ng/ml)	CV (%)
Sample 1	10	2.39	0.14	5.98
Sample 2	14	5.37	0.21	3.93
Sample 3	11	14.33	0.45	3.12

Linearity

Dilution:	Sample 1 (calculated, ng/ml)	Dilution:	Sample 2 (calculated, ng/ml)
1:10	13.40	1:600	733.0
1:20	13.96	1:1200	749.8
1:40	13.50	1:2400	759.8
1:80	13.47	1:4800	756.7
1:160	13.35	1:9600	797.3
1:320	13.50		
AV / 1SD	13.53 / 0.219	AV / 1SD	759.3 / 23.6

AV = Average Value , SD = Standard Deviation

Recovery

The recovery of the recombinant hGH yielded in a buffer matrix 100%. In different human-sera the recovery was on average 94 % of the hypothetical expected amount. Recovery of 10ng/ml recombinant hGH in serum matrices in reffered to buffer.

	Sample 1	Sample 2	Sample 3
Recovery [%]	96.46	92.03	91.4

LITERATUR

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SUMMARY – hGH-SENSITIVE ELISA RMEE022

Reagents:	Reconstitution:	Dilution/ Mixing:
Standards A-E	in 750 µL Dilution Buffer **VP**	
Control Serum KS	in 100 µL Dilution Buffer **VP**	**1:26 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: with **Dilution Buffer VP** generally **dilution of 1:26** (e.g. dilute 10 µL serum with 250 µL Dilution Buffer VP). Use **100 µL** per determination.		
Before assay procedure bring all **reagents** to **room temperature**.		

Proposal of Assay Procedure for double determinations

Pipette	Reagents	Position
100 µL	Dilution Buffer **VP (blank)**	A1/2
100 µL	Standard **A** (**0.05 ng/ml**)	B1/2
100 µL	Standard **B** (**0.15 ng/ml**)	C1/2
100 µL	Standard **C** (**0.30 ng/ml**)	D1/2
100 µL	Standard **D** (**0.6 ng/ml**)	E1/2
100 µL	Standard **E** (**1.0 ng/ml**)	F1/2
100 µL	Control Serum **KS** (1:26 diluted)	G1/2
100 µL	Sample Dilution	Pipette sample in the rest of the wells according the requirements
Cover the wells with the sealing tape.		

Sample Incubation: **2 h at RT**, ≥ **350 rpm**

3x 250 µL	Aspirate the contents of the wells and **wash** 3x with 250 µL each Wash Buffer **WP/well**	in each well
100 µL	**Antibody Conjugate AK**	in each well

AK Incubation 0.5h at RT, ≥350 rpm

100µl	Enzyme conjugate **EK, without washing the wells (!)** – add to the previously pipetted AK-solution **thereto,** thereby simultaneously mixing or mix shortly through cautious tapping on the MTP. **Attention: high filled volume of the wells!**	in each well

EK Incubation 0.5h at RT, without shaking

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

Substrate S Incubation: 15 min. in the dark at RT

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

Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, Belgium
http://www.gentaur-worldwide.com