hGH - ELISA
(human Growth Hormone; Somatropin)

Enzyme Immunoassay for the Quantitative Determination of

human Growth Hormone

Product-Code: E02
(96 Determinations)

For In Vitro Diagnostic Use

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INTENDED USE

An enzyme immunoassay for the quantitative *in-vitro diagnostic* measurement of levels of human growth hormone in serum and plasma. Human growth hormone measurements are used in the diagnosis and treatment of disorders involving the anterior lobe of the pituitary gland.

SUMMARY AND EXPLANATION

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-glycosylated 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 modified 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 of hGH are also manifold. These 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 spontaneus 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 Mediagnost hGH ELISA E02 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

1. For In Vitro Diagnostic Use
2. For professional use only.
3. **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.
4. Before use, all kit components should be brought to room temperature at 68-77°F (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.
5. Do not mix reagents of different lots. Do not use expired reagents.
6. The microplate contains snap-off strips. Unused wells must be stored at 35.6-46.4°F (2-8°C) in the sealed foil pouch and used in the frame provided.
7. **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) antibodies. 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.
8. Stop solution contains 0.2 M Sulfuric Acid (H₂SO₄)
   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.
9. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
10. Use separate pipette tips for each sample, control and reagent to avoid cross contamination.
11. 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.
12. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
13. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
14. Some reagents contain <0.01% 2-Methyl-4-isothiazolin-3-one Solution and <0.01% 5-chloro-2-methyl 2H isothiazol-3-one and 2-methyl-2H-isothiazol-3-one as preservatives. In case of contact with eyes or skin, flush immediately with water.
15. 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) **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) **Standards (A-E)**, 750 µl each, ready-to-use. Contain recombinant hGH (NIBSC 98/574). Standard values are between 1 – 25 ng/ml (1, 5, 10, 15 and 25 ng/ml) hGH, 20 µl are used per well in the assay.

3) **Dilution Buffer (VP)**, 30 ml, ready-to-use, please use for the sample dilution, if necessary.

4) **Control Sera (KS1&KS2)**, 750 µl each, ready-to-use, contain human serum. The hGH target values and the respective ranges are given on the vial labels. The dilution of the Control Sera should be according to the dilution of the respected samples, generally undiluted 20µl/well.

5) **Antibody Conjugate (AK)**, 12 ml, ready-to-use solution, contains rabbit biotinylated anti-hGH antibody (100µl/well).

6) **Enzyme Conjugate (EK)**, 12 ml, ready-to-use solution, contains HRP (Horseradish-Peroxidase)-labelled Streptavidin (100µl/well).

7) **Washing Buffer (WP)**, 50 ml, 20 X concentrated solution;

8) **Substrate (S)**, 12 ml, ready for use, horseradish-peroxidase-(HRP)-substrate, stabilised H₂O₂-Tetramethylbenzidine.

9) **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

1. Precision pipettes and multichannel pipettes with disposable plastic tips
2. Distilled or deionized water for dilution of the **Washing Buffer (WP)**
3. Vortex-mixer
4. Microtiter plate shaker (350 rpm)
5. Microtiter plate washer (recommended)
6. Micro plate reader ("ELISA-Reader") with filter for 450 and ≥ 590 nm
REAGENT PREPARATION
Bring all reagents to room temperature at 68-77°F (20 - 25°C) before use.

Wash Solution: Add distilled/deionized water to the complete contents of the (20x) concentrated Wash Solution (50 ml) into a graduated flask and fill to a final volume of 1000 ml, or dilute 1:20 only according to requirements. Attention: After dilution the Washing Buffer is only 4 weeks stable, please dilute only according to requirements.

TECHNICAL NOTES
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 for 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.

STORAGE CONDITIONS
The microtiter plate wells and all undiluted reagents are stable until the expiry date if stored in the dark at 35.6-46.4°F (2-8°C). Store the unused seal strips and microtiter wells together with the desiccant at 35.6-46.4°F (2-8°C).

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

SPECIMEN COLLECTION, PREPARATION AND STORAGE
Serum as well as plasma samples are suitable (significant deviation of hGH levels in corresponding Serum, Heparin-, Citrate-, 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 -4°F, (-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.

In most determinations (serum or plasma samples, and no extreme values expected) the sample size of 20 µl, undiluted, is optimally suitable. The hGH concentrations may be completely different in body fluids of human origin other than serum or cell culture supernatants.

If necessary samples can be diluted in Dilution Buffer VP. For the determination of samples with very low hGH-levels the Mediagnost hGH-Sensitiv ELISA E022 might be better suited (Sensitivity 0.0016 ng/ml ≈ 1.6 pg/ml).

ASSAY PROCEDURE
When performing the assay, the Standards, Control Sera and the samples should be pipette as fast as possible (e.g., <15 minutes). To avoid distortions due to differences in incubation times, the 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.
ASSAY PROCEDURE

NOTES: All determinations (Standards, Control Sera and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended. The assay must be performed at room temperature.

Please pipette on before in all needed wells 100 µl of Dilution Buffer VP.

1) Add additional 20 µl Dilution Buffer VP in wells A1/A2 (blank).

2) Pipette in positions B1/2 20 µl of Standard A (1 ng/ml)
Pipette in positions C1/2 20 µl Standard B (5 ng/ml),
Pipette in positions D1/2 20 µl Standard C (10 ng/ml),
Pipette in positions E1/2 20 µl Standard D (15 ng/ml),
Pipette in positions F1/2 20 µl Standard E (25 ng/ml).

To control the correct accomplishment 20 µl of the undiluted (or in respective dilution rate of the sample) Control Sera KS1 and KS2 can be pipetted in positions G1/2 and H1/H2.

Pipette 20 µl of the undiluted 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 3 times with 300 µl Washing Buffer WP / well.

5) Following the last washing step, pipette 100 µl of the Antibody Conjugate AK in each well and incubate 30 minutes (shake at 350 rpm).

6) Subsequently –without a washing step! - pipette 100 µl of the Enzyme-Conjugate in each well and incubate additional 30 minutes without shaking.
The solutions should now be mixed through the addition; slight shaking or tapping on the border of the microtiter plate could support this. Attention: The risk of the cross contamination is increased through the high filled volume of the wells.

7) After incubation, wash the wells 3 times with Washing Buffer (WP) as described in step 4.

8) Pipette 100 µl of the TMB Substrate Solution S in each well. Incubate the plate for 15 minutes in the dark at room temperature.

9) Stop the reaction by adding 100 µl of Stopping Solution (SL)

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 (6), was used as standard material. The International Standard 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 should be below 0.25, and the absorbance of standard E should be above 1.00. Samples, which yield higher absorbance values than Standard E, are beyond the standard curve. For reliable determinations these samples should be re-tested with a higher dilution.

The standards provided contain the following concentration of hGH:

<table>
<thead>
<tr>
<th>Standard</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/ml</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>µIU/ml</td>
<td>3</td>
<td>15</td>
<td>30</td>
<td>45</td>
<td>75</td>
</tr>
</tbody>
</table>

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.
4) Recommendation: Calculation of standard curve should be done by using a computer programme, 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 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.

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**Example of Typical Standard Curve**
The following data is for demonstration only and cannot be used in place of data generation at the time of assay.

<table>
<thead>
<tr>
<th>Standard</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/ml</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Extinction</td>
<td>0.19</td>
<td>0.76</td>
<td>1.3</td>
<td>1.77</td>
<td>2.34</td>
</tr>
</tbody>
</table>

**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 (6), 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.

<table>
<thead>
<tr>
<th></th>
<th>female</th>
<th>male</th>
</tr>
</thead>
<tbody>
<tr>
<td>number</td>
<td>54</td>
<td>50</td>
</tr>
<tr>
<td>median [ng/ml]</td>
<td>0.81</td>
<td>0.28</td>
</tr>
<tr>
<td>minimal concentration [ng/ml]</td>
<td>0.19</td>
<td>0.15</td>
</tr>
<tr>
<td>maximal concentration [ng/ml]</td>
<td>10.15</td>
<td>4.34</td>
</tr>
</tbody>
</table>

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.
LIMITATIONS OF PROCEDURE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.

Specimen collection has a significant effect on the test results. See SPECIMEN COLLECTION AND STORAGE for details.

Reproducible results depend on careful pipetting, observation of incubation periods and temperature, as well as rinsing the test strips and thorough mixing of all prepared solutions.

Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled evenly with Washing Solution, and that there are no residues in the wells.

Instructions for using appropriate photometers are to be observed; check adjustment of proper wavelength and reference wavelength respectively.
PERFORMANCE CHARACTERISTICS

Sensitivity
The analytical sensitivity of the hGH ELISA E02 yields 0.25 ng/ml (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 200 ng/ml prolactin solution in this assay, no cross reactivity was detected.

Reproducibility

<table>
<thead>
<tr>
<th>Intra-Assay-Variation</th>
<th>Number of determinations</th>
<th>Mean value (ng/ml)</th>
<th>Standard deviation (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>16</td>
<td>5.84</td>
<td>0.27</td>
<td>4.70</td>
</tr>
<tr>
<td>Sample 2</td>
<td>16</td>
<td>14.92</td>
<td>0.55</td>
<td>3.68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inter-Assay-Variation</th>
<th>Number of determinations</th>
<th>Mean value (ng/ml)</th>
<th>Standard deviation (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>10</td>
<td>2.57</td>
<td>0.15</td>
<td>5.68</td>
</tr>
<tr>
<td>Sample 2</td>
<td>10</td>
<td>5.80</td>
<td>0.31</td>
<td>5.38</td>
</tr>
<tr>
<td>Sample 3</td>
<td>10</td>
<td>10.86</td>
<td>0.72</td>
<td>6.62</td>
</tr>
</tbody>
</table>

Linearity

<table>
<thead>
<tr>
<th>Dilution:</th>
<th>Sample 1 (calculated, ng/ml)</th>
<th>Dilution:</th>
<th>Sample 2 (calculated, ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>undiluted</td>
<td>14.1</td>
<td>1:30</td>
<td>845.3</td>
</tr>
<tr>
<td>1:2</td>
<td>16.5</td>
<td>1:60</td>
<td>778.5</td>
</tr>
<tr>
<td>1:4</td>
<td>16.2</td>
<td>1:120</td>
<td>732.4</td>
</tr>
<tr>
<td>1:8</td>
<td>16.0</td>
<td>1:240</td>
<td>664.1</td>
</tr>
<tr>
<td>1:16</td>
<td>15.1</td>
<td>1:480</td>
<td>738.0</td>
</tr>
<tr>
<td>1:32</td>
<td>14.5</td>
<td>1:960</td>
<td>733.4</td>
</tr>
<tr>
<td>1:64</td>
<td>14.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AV / 1SD / VC% = 15.3 / 0.92 / 5.98
AV / 1SD / VC% = 748.6 / 60.0 / 8.01

AV = Average Value, SD = Standard Deviation, VK = Variation coefficient %
Recovery
The recovery of the recombinant hGH yielded in a buffer matrix 100%. Recovery of 10 ng/ml recombinant hGH in serum matrices referred to buffer.

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery [%]</td>
<td>96.4</td>
<td>92</td>
</tr>
</tbody>
</table>

COMPARISON STUDIES
MEDIAGNOST test hGH test is adapted to and has therefore the identical results as the in-house assay Tuebingen compared to commercially available tests in Hauffa et al. (2004). This study evaluated 699 peak serum samples of 258 mal and 124 female children for human growth hormone and compares seven different assays. The concordance of the Tuebingen in house assay and therefore the Mediagnost ELISA between two commercially available reference assays was highest with $p_c >0.86$. 
REFERENCES


6) Address NIBSC: National Institute for Biological Standards and Controls, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.


### SUMMARY: Mediagnost hGH ELISA (E02)

#### Reagents preparations

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Bring to room temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>The ready for use reagents:</td>
<td></td>
</tr>
<tr>
<td><strong>Standard A – E</strong> (each 750 µl),</td>
<td></td>
</tr>
<tr>
<td><strong>Control sera KS1 &amp; KS2</strong> (750 µl),</td>
<td></td>
</tr>
<tr>
<td><strong>Antibody conjugate AK</strong> (12ml),</td>
<td></td>
</tr>
<tr>
<td><strong>Enzyme conjugate EK</strong> (12ml)</td>
<td></td>
</tr>
<tr>
<td><strong>Washing buffer</strong> (50 ml)</td>
<td>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)</td>
</tr>
</tbody>
</table>

#### Samples

| Dilution is generally not necessary, just use 20 µl per single determination. |

#### Proposal of Assay Procedure for double determinations:

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Reagents</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µl</td>
<td>Dilution Buffer VP</td>
<td>Pipette in all required number of wells</td>
</tr>
<tr>
<td>20 µl</td>
<td>Dilution buffer VP as blank</td>
<td>A1 and A2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Standard A (1 ng/ml)</td>
<td>B1 and B2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Standard B (5 ng/ml)</td>
<td>C1 and C2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Standard C (10 ng/ml)</td>
<td>D1 and D2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Standard D (15 ng/ml)</td>
<td>E1 and E2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Standard E (25 ng/ml)</td>
<td>F1 and F2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Kontrollserum KS1</td>
<td>G1 and G2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Kontrollserum KS2</td>
<td>H1 and H2</td>
</tr>
<tr>
<td>20 µl</td>
<td>Sample</td>
<td>Pipette sample in the rest of the wells according the requirements (Pipetting Control = colour turns red!)</td>
</tr>
</tbody>
</table>

Cover the wells with the sealing tape.

#### Incubation: 1 h at RT, 350 rpm

3 x 300 µl Aspirate the contents of the wells and wash 3 x with 300 µl Wash Buffer WP in each well

100 µl Antibody conjugate AK in each well

#### Incubation: 30 min at RT, 350 rpm

100 µl 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

#### Incubation: 30 min at RT, without shaking.

3 x 300 µl Aspirate the contents of the wells and wash 3 x with 300 µl Wash Buffer WP in each well

100 µl Substrate solution S in each well

#### 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.