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Print Protocol

Print Changes to Previous Version

hGH ELISA

Version 08

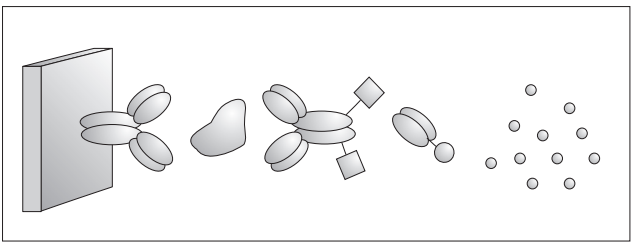
Content version: January 2019

Colorimetric enzyme immunoassay for the quantitative determination of secreted human growth hormone (hGH)

Cat. No. 11 585 878 001 192 tests

Store the kit at +2 to +8°C.

Test principle



Contents

Kit Contents

1.	**hGH (human Growth Hormone),** recombinant protein (22 kDa) from *E. coli*, for exact content see lot-specific label imprint. Lyophilizate, stabilized Purity: >98% (SDS PAGE)	approx. 5 ng	blue cap
2.	**Anti-hGH-DIG,** polyclonal antibody to hGH (from sheep) that is conjugated to digoxigenin. Lyophilizate, stabilized	50 µg	white cap
3.	**Anti-DIG-POD,** polyclonal antibody to digoxigenin (from sheep) that is conjugated to peroxidase. Lyophilizate, stabilized	10 U	red cap
4.	**POD Substrate,** ABTS[1], ready-to-use solution, stabilized	100 ml	green cap
5.	**Substrate Enhancer.** Powder *Use the substrate enhancer only if the hGH concentration is low!*	150 mg	green cap
6.	**Washing Buffer Concentrate (10×),** PBS (phosphate buffered saline), containing Tween[2] 20, 10× conc. solution	120 ml	colorless cap
7.	**Sample Buffer,** PBS containing blocking reagents, ready-to-use solution	2× 100 ml	red cap
8.	**Microplate,** strip frame with 12 modules of 8 wells pre-coated with a polyclonal antibody to hGH (from sheep) and postcoated with blocking reagent. Shrink-wrapped, with a desiccant capsule	2 plates (24× 8 wells)	foil bag
9.	**Self-adhesive plate cover foil.**	6 foils	

Stability

If stored at +2 to +8°C the kit is stable until the expiration date printed on the label. For stability of kit components and working solutions see section 6.2.

Advantages of hGH ELISA

- **standardized** (allows direct comparison of data from different sets of experiments even when kits from different production lots are used)
- **highly sensitive** [approx. 20 times more sensitive than isotopic hGH assays (see fig. 2)]
- **specific** (no cross reaction with rat growth hormone)
- **fast** [measures hGH expression already 18 hours post-transfection (see fig 2); requires only approx. 4 hours from start to finish]
- **no leaching** (coating of microplates follows a proprietary, optimized protocol)
- high intra- and inter-assay reproducibility
- **comparable** (to the non-radioactive determination of CAT or β-Gal levels using the CAT ELISA or β-Gal ELISA)
- **easy to perform** (follows a standard ELISA protocol)
- **function-tested**

1. Introduction

Human Growth Hormone (hGH; somatotropin) is a 22 kDa, 191 amino acid, polypeptide hormone synthesized and secreted by cells of the anterior pituitary. The hGH gene contains five exons and is the best characterized of five members of a gene family (1).

Gene expression in transfected mammalian cells is generally studied by linking a promoter sequence to mammalian or bacterial genes encoding an easily detectable "reporter"-protein such as human growth hormone, chloramphenicol acetyl transferase (CAT), β-galactosidase (β-Gal) or luciferase (2). The hGH assay system differs from most of the other commonly used reporter proteins such as CAT, β-galactosidase or luciferase in one important respect: hGH is a secreted protein and is measured using samples of culture medium supernatants thus (i) avoiding the necessity to lyse cells (ii) allowing continuous monitoring of transient expression kinetics and (iii) allowing the use of the cells for other purposes e. g. RNA isolation (2). Additionally, hGH is a mammalian gene which may contribute to the apparent high stability of the hGH mRNA in most mammalian cells. The expression of reporter protein correlates directly to changes in the level of hGH mRNA (3).

Using the standard isotopic protocol, secretion of hGH protein into the culture medium supernatant can be monitored approx. 24 hours post transfection, depending on the cell type and the sensitivity of the assay used. Usually, hGH is quantified by an immunoradiometric assay (IRMA) resulting in a linear range of approx. 0.1 to 50 ng hGH/ml (2). Various vectors, *e.g.*, pXGH5, pØGH containing polylinkers adjacent to the hGH sequence for insertion of promoters, are commonly used (3, 4). In transiently transfected mouse L cells, the immunoradiometric hGH assay was described to be at least 10 times more sensitive than the CAT assay system (2). It should be noted that the hGH gene may not be entirely silent in terms of regulatory signals. In some cases, depending on the chosen cell line, the immunoradiometric hGH assay was reported to cross-react with rat growth hormone which is also secreted into the medium supernatant resulting in an elevated background signal.

2. Application

The hGH ELISA is used to quantitatively measure the expression of hGH released into the cell culture supernatant of eukaryotic cells transfected with a plasmid containing a reporter gene encoding for hGH.

3. Test Principle

The hGH ELISA is based on the sandwich ELISA principle. Antibodies to hGH (anti-hGH) are prebound to the surface of the microplate modules (MP modules; see fig. 1). Following transfection of cells, the culture supernatant, which contains secreted hGH, is added to the wells of the MP modules. All hGH contained in the medium binds specifically to the anti-hGH antibodies bound to the microplate surface (fig. 1, step 1). Next, a digoxigenin-labeled antibody to hGH (anti-hGH-DIG) is added and binds to hGH (fig. 1, step 2). In the following step, an antibody to digoxigenin conjugated to peroxidase (anti-DIG-POD) is added and binds to digoxigenin (fig. 1, step 3). In the final step, the peroxidase substrate ABTS is added (fig. 1, step 4). The peroxidase enzyme catalyzes the cleavage of the substrate yielding a colored reaction product. The absorbance of the sample is determined using a microplate (ELISA) reader and is directly correlated to the level of hGH present in the medium supernatant. The sensitivity of the assay can be enhanced by using the peroxidase substrate (ABTS) with substrate enhancer.

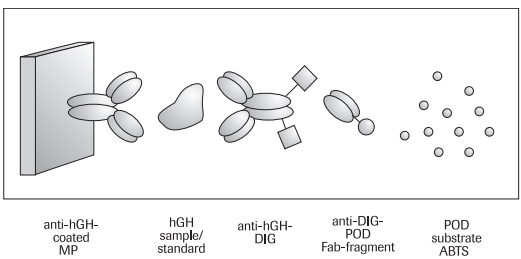


Fig. 1: Test principle

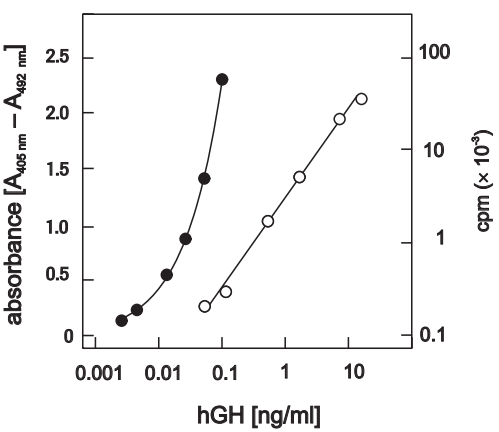


Fig. 2: Comparison of an immunoradiometric hGH assay (IRMA; ○) versus the hGH ELISA (●), Various dilutions of hGH standard were assayed following a standard sandwich IRMA procedure using two monoclonal antibodies, each specific for a different and distinct epitope. One antibody is radiolabeled [^{125}I] while the other antibody is coupled to biotin. The incubation of the two antibodies and the sample containing hGH with an avidin-coated polystyrene bead allows the quantification of bound hGH. The non-isotopic hGH ELISA was performed as described. (**Note:** in a non-logarithmic presentation the hGH ELISA produces a linear calibration curve; see fig 4).

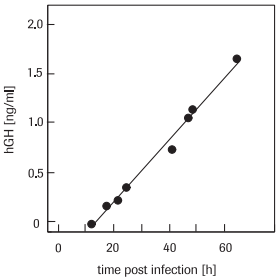


Fig. 3: HeLa cells were grown in culture medium supplemented with 10% fetal calf serum (FCS). Cells (0.8×10^6) were seeded in 6 cm dishes for 14 hours before transfection. Cells were transfected with 5 µg of the hGH-expressing vector pXGH5 using the transfection reagent DOTAP* (6 µg DOTAP per 1 µg DNA) in medium, supplemented with 10% FCS, as described (5). The release of hGH into the culture supernatant was determined using the hGH ELISA. To achieve measurement of hGH in the linear range of the assay, aliquots of the culture supernatants were diluted with sample buffer
(solution 7). The amount of hGH in the supernatant was determined by comparing absorbance values with those of a calibration curve.

4. Assay Characteristics

Sensitivity	≥ 5 pg/ml (≥ 1 pg/well)
Specificity	The hGH ELISA specifically detects human Growth Hormone (hGH). It does not cross-react with rat Growth Hormone. Cross reactivity with the human homologues TSH, FSH, and LH has not been tested.
hGH Standards	The hGH protein from *E. coli*, included in the kit for the purpose of compiling a standard calibration curve, is provided with lot-specific content data as determined by immunoassay. The purity is > 98% (SDS PAGE).
Sample Material	Cell culture supernatants from transfected cells, no less than approx. 18 hours post transfection.
Assay Time	Approx. 4 hours

5. Sample Preparation

Cell culture supernatants may be used directly as sample material in the hGH ELISA, without centrifugation. When testing culture supernatants with high hGH concentrations, dilute the medium with sample buffer (solution 7) or PBS and add 200 µl of these dilutions to the wells. hGH is stable in culture medium for several months at −15 to −25°C if stored without repeated freezing and thawing, and for several days at +2 to +8°C.

6. Preparation of Solutions

6.1 Material and Reagents

All necessary reagents are contained in the kit.
Redist. water should always be used for reconstitution and dilution purposes.

6.2 Preparation of Working Solutions

Solution 1 — **hGH Stock Solution (bottle 1)**
Reconstitute the lyophilizate in 0.5 ml redist. water. The resulting concentration is calculated using the lot-specific information, given on the label (final conc. approx 10 ng/ml). This solution is used for the preparation of hGH standards for establishing a hGH calibration curve (see 7.2: preparation of hGH standards) The reconstituted solution is stable for 2 months at +2 to +8°C. For long term storage, store in aliquots at or below −15 to −25°C.

Solution 2 — **Anti-hGH-DIG (bottle 2)**
Reconstitute the lyophilizate in 0.5 ml redist. water (final conc. 100 µg/ml).
The reconstituted solution is stable for two months at +2 to +8°C. For long term storage, store in aliquots at or below −15 to −25°C.

Solution 2a — **Anti-hGH-DIG, working dilution**
To prepare anti-hGH-DIG working dilution, dilute the reconstituted anti-hGH-DIG solution (100 µg/ml) with sample buffer (solution 7) to a final conc. of 1 µg/ml (*e.g.*, 100 µl of reconstituted anti-hGH-DIG solution + 9.9 ml of solution 7 for 50 wells). The working dilution should be prepared freshly before use and should not be stored.

Solution 3 — **Anti-DIG-POD (bottle 3)**
Reconstitute the lyophilizate in 0.5 ml redist. water (final conc. 20 U/ml). The reconstituted solution is stable for 6 months at +2 to +8°C. *Do not freeze! Do not add sodium azide!*

Solution 3a — **Anti-DIG-POD, working dilution**
To prepare anti-DIG-POD, working dilution, dilute the reconstituted anti-DIG-POD solution (20 U/ml) with sample buffer (solution 7) to a final conc. of 200 mU/ml (*e.g.*, 100 µl of reconstituted anti-DIG-POD solution + 9.9 ml of solution 7 for 50 wells). The working dilution should be prepared freshly before use and should not be stored.

Solution 4 — **POD Substrate (bottle 4)**
Ready-to-use solution. The solution is stable until the expiry date given on the label if stored at +2 to +8°C.

Solution 5 **POD Substrate containing Substrate Enhancer (bottles 4 and 5)**
If a low hGH concentration in the sample is expected, add 1 mg of substrate enhancer (bottle 5) per ml of solution 4 and mix by stirring for 30 min at +15 to +25°C. The solution is stable for only 2 hours and should therefore be prepared immediately before use. Use the substrate enhancer only if the hGH concentration is low!

Solution 6 **Washing Buffer, 1× (bottle 6)**
To prepare a ready-to-use washing buffer, mix 1 part 10× washing buffer concentrate (bottle 6) with 9 parts of redist. water. The reconstituted solution is stable for 6 months at +2 to +8°C.

Solution 7 **Sample Buffer (bottle 7)**
The solution is ready-to-use. Mix thoroughly before use. After opening the bottle we recommend storing the solution in aliquots at −15 to −25°C, since it does not contain a preservative agent. *Do not add sodium azide.*

6.3 Microplate

Use only the microplate (MP) modules required for the particular experiment. Close the foil bag containing the remaining MP-modules and the dessiccant capsule tightly with adhesive tape.
The anti-hGH-coated MP-modules are ready-to-use and need not to be rehydrated before use.
Once, the foil bag is opened, MP modules are stable for a minimum of 2 weeks if stored dessicated at +2 to +8°C.

7. Example: Measurement of hGH

7.1 General Recommendations

Most of the available ELISA readers reach their absorbance maximum at approx. 2 to 2.5 optical density units (OD). Measurement of supernatants with high hGH concentrations therefore requires dilution of the supernatants with sample buffer (solution 7).
The use of the substrate enhancer with POD substrate ABTS (solution 5) approximately doubles the sensitivity of the assay (fig. 3). When the test is first performed under non-optimal conditions *e.g.*, the POD substrate ABTS without substrate enhancer (solution 4) may be removed. After washing each well two times with washing buffer (solution 6), incubate with POD substrate ABTS with substrate enhancer (solution 5) as described above to increase sensitivity. Using the same procedure, an incubation with substrate buffer with substrate enhancer may be followed by an incubation with POD substrate solution without substrate enhancer to improve detection range.
Prolonged incubation of the samples with the peroxidase substrate ABTS (*e.g.*, overnight at +2 to +8°C) can produce a non-linear calibration curve and is therefore only recommended for qualitative analysis of hGH expression.
Since the POD substrates (solutions 4, 5) are slightly colored, leave one well free in order to determine the blank (baseline) value. Add POD substrate to this well for use as a reference when measuring the MP modules in the ELISA reader. Most readers can be programmed to automatically substract the reference (blank) value from the values of the other samples.

(used to produce a calibration curve for the hGH protein)
The hGH standard working dilutions should be prepared freshly before use and should not be stored.

Mix 40 µl hGH stock solution (**approx**. 10 ng/ml, solution 1) with 960 µl sample buffer (solution 7) (final conc. **approx**. 400 pg/ml; depending on the lot-specific content).

Prepare the standard dilution series in reaction tubes in 1:2 dilution steps as described in the table below. To avoid carry over of the higher concentrated solution to the lower concentrated samples, use a fresh pipette tip for each dilution step.

To ensure that the measurements and the calibration curve are accurate, two samples of each concentration must be prepared.

Step	**hGH working dilution**	**Add sample buffer** (solution 7)	**Approximate hGH concentration (pg/ml)**
0	0	1000 µl	0
1	1000 µl	0	400
2	500 µl of step 1	500 µl	200
3	500 µl of step 2	500 µl	100
4	500 µl of step 3	500 µl	50
5	500 µl of step 4	500 µl	25
6	500 µl of step 5	500 µl	12.5

200 µl of each dilution is needed per well. Each dilution must be measured in duplicate (see section 7.4).

7.3 Pipetting Scheme for the Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Bl	P1	P1								
B	S1	S1	P2	P2								
C	S2	S2										
D	S3	S3										
E	S4	S4										
F	S5	S5										
G	S6	S6										
H	S7	S7									P32	P32

Legend:
Bl = blank (= POD substrate)
S1-S7 = hGH standard dilutions
P1-P32 = samples 1-32

Important: All reagents should be fully equilibrated to +15 to +25°C before starting the test. Reagents from kits with different lot Nos. must not be used in one test series.

ELISA Procedure

To ensure that the measurements are accurate, we recommend measuring two samples of each culture supernatant.

- Use only the microplate (MP) modules required for the particular experiment and place them in the frame in the correct orientation. (Correct fitting ensures a tight support of the MP modules). MP modules are ready-to-use and need not to be rehydrated prior to addition of the samples.
- Pipette 200 µl of hGH standard working dilution (prepared as described in section 7.2) per well, **or** 200 µl culture supernatant per well.
  Cover the MP modules with the cover foil and incubate for 1 h at +37°C.
- Remove the solution thoroughly. Rinse wells 5 times with 250 µl of washing buffer (solution 6) for 30 s each and remove washing buffer carefully.
- Pipette 200 µl of anti-hGH-DIG working dilution (solution 2a) into each well, cover the MP modules with the cover foil and incubate for 1 h at +37°C.
- Remove the solution thoroughly. Rinse wells 5 times with 250 µl of washing buffer (solution 6) for 30 s each and remove washing buffer carefully.
- Pipette 200 µl of anti-DIG-POD working dilution (solution 3a) into each well, cover the MP modules with the cover foil and incubate for 1 h at +37°C.
- Remove the solution thoroughly. Rinse wells 5 times with 250 µl of washing buffer (solution 6) for 30 s each and remove washing buffer carefully.

Substrate Reaction

- Pipette 200 µl of POD substrate (solution 4) or POD substrate with substrate enhancer (solution 5) into each well and incubate at +15 to +25°C until color development (green color) is sufficient for photometric detection (10-30 min).
- Measure the absorbance of the samples at 405 nm with a reference wavelength at approx. 490 nm, using a microplate (ELISA) reader (*e.g.*, EAR 340 ATTC, SLT Lab Instruments).

Note: Shaking of microplates at 250 rpm during incubation with substrate solution can be employed to shorten the incubation period, but is not essential. If shaking is not carried out, gently tap on the side of the microplate before measuring absorbance to ensure a homogeneous distribution of the colored reaction product.

Interpretation

Upon completion of the experimental procedure, calculate the exact hGH concentration (pg/ml) of the calibration standards. Plot the absorbance values obtained on the y-axis against the lot-specific standard concentrations on the x-axis. This results in a linear calibration curve (for an example see fig. 4).

The hGH concentration of unknown samples can then be determined by plotting the observed absorbance values on the y-axis, extrapolating to meet the calibration curve and reading the hGH concentration of the x-axis. To obtain reliable results, the absorbance values of the unknown sample should lie within the linear portion of the calibration curve.

Note: A separate calibration curve must be established for each series. We recommend that one experimental series be performed on one microplate. When more than one microplate is used in one series, a calibration must be carried out on each plate.

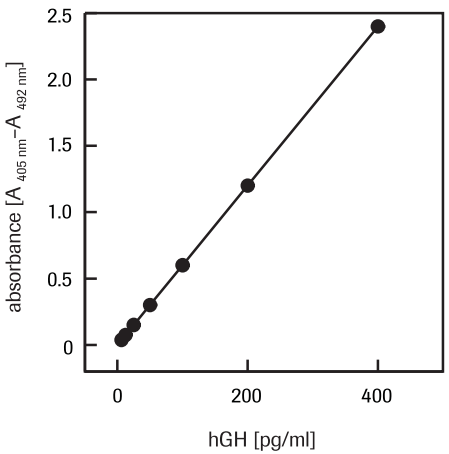


Fig. 4: A typical calibration curve using POD substrate ABTS after a 15 min color reaction.

8. References

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2 Alam, J. & Cook, J.L. (1990) *Anal. Biochem.* **188**, 245.
3 Ausubel, F.M. et al. (1992) In: Current protocols in molecular biology; publ. John Wiley & Sons, Vol. 1, suppl. 14, 9.7.
4 Selden, R.F. et al. (1986) *Mol. Cell Biol.* **6**, 3173.
5 Doppler, C. et al. (1992) *AIDS Res. Human Retroviruses* **8**, 245.
6 Shewchuk, B. M. et al. (2002) *PNAS* **99**, 11784-11789.

* *available from Roche Diagnostics*

Changes to previous version

Editorial changes.

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9. Ordering Information

Product	Pack size	Cat. No.
DOTAP Liposomal Transfection Reagent	400 µl (1 × 0.4 mg) 2 ml (5 × 0.4 ml) (5 ¥ 0.4 mg)	11 811 177 001 11 202 375 001
DOSPER Liposomal Transfection Reagent	400 µl (1 × 0.4 mg) 2 ml (5 × 0.4 ml) (5 × 0.4 mg)	11 811 169 001 11 781 995 001
Anti-CAT-coated Microplate, transparent	192 tests	11 465 074 001
CAT ELISA	1 kit (192 tests)	11 363 727 001
β-Gal ELISA	1 kit (192 tests)	11 539 426 001
β-Gal Reporter Gene Assay, chemiluminescence	1 kit (500 assays, microplate format 250 assays, tube format)	11 758 241 001
β-Gal Staining Set	1 Set (for 100 tests in 3.5 cm dishes)	11 828 673 001
SEAP Reporter Gene Assay, chemiluminescence	1 kit (500 assays, MP format 250 assays, tube format)	11 779 842 001
X-Gal	100 mg 250 mg 1 g 2.5 g	11 680 293 001 10 651 745 001 10 745 740 001 10 703 729 001
Luciferase Reporter Gene Assay, high sensitivity	200 assays 1,000 assays	11 669 893 001 11 814 036 001
Reporter Gene Assay Lysis Buffer	50 ml	11 897 675 001
Cell Proliferation Reagent WST-1	2,500 tests	11 644 807 001
FuGENE® 6 Transfection Reagent	0.4 ml 1 ml 5 × 1 ml	11 815 091 001 11 814 443 001 11 988 387 001
FuGENE® HD Transfection Reagent	0.4 ml (up to 120 transfections) 1 ml (up to 300 transfections) 5 × 1 ml (up to 1,500 transfections)	04 709 691 001 04 709 705 001 04 709 713 001
Geneticin[1)] (G418)	1 g 5 g	11 464 981 001 11 464 990 001
Hygromycin B	1 g (20 ml)	10 843 555 001

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10. Quick reference protocols

10.1 Required solutions

Solution	Content	Used for
1	**hGH Stock Solution** (bottle 1, blue cap): Reconstitute in 0.5 ml redist. water; prepare hGH standards for the construction of a calibration curve [see section 7.2 (10.2)]	section 7.2 (10.2)
2	**anti-hGH-DIG** (bottle 2, white cap): Reconstitute in 0.5 ml redist. water.	solution 2a
2a	**anti-hGH-DIG, working dilution:** dilute solution 2 with solution 7 to a final conc. of 1 µg/ml (*e.g.*, 100 µl reconst. solution + 9.9 ml of solution 7)	step 3
3	**anti-DIG-POD** (bottle 3, red cap): Reconstitute in 0.5 ml redist. water. (20 U/ml)	solution 3a
3a	**anti-DIG-POD, working dilution:** dilute solution 3 with solution 7 to a final conc. of 200 mU/ml (*e.g.*, 100 µl reconst. solution + 9.9 ml of solution 7)	step 5
4	**POD Substrate** (bottle 4, green cap): ready-to-use solution	step 7
5	**POD Substrate containing substrate enhancer:** Add 1 mg of substrate enhancer (bottle 5, green cap) per ml of solution 4 *Use the substrate enhancer only if the hGH concentration is low!*	step 7
6	**Washing Buffer, 1×** (bottle 6, colorless cap): Mix 1 part of washing buffer concentrate, 10× (bottle 6) with 9 parts of redist. water	steps 2, 4 and 6
7	**Sample Buffer** (bottle 7, red cap)**:** ready-to-use solution	solutions 2a, 3a, section 7.2 (10.2)

10.2 Preparation of hGH standards

Mix 40 µl hGH stock solution (**approx**. 10 ng/ml, solution 1) with 960 µl sample buffer (solution 6) (final conc. **approx**. 400 pg/ml, depending on the lot-specific content).

Prepare the standard dilution series in reaction tubes in 1 : 2 dilution steps as described in the table below. To avoid carry over of the higher concentrated solution to the lower concentrated samples, use a fresh pipette tip for each dilution step.

Step	hGH working dilution	Add sample buffer (solution 7)	Approximate hGH concentration (pg/ml)
0	0	1000 µl	0
1	1000 µl	0	400
2	500 µl of step 1	500 µl	200
3	500 µl of step 2	500 µl	100
4	500 µl of step 3	500 µl	50
5	500 µl of step 4	500 µl	25
6	500 µl of step 5	500 µl	12.5

200 µl of each dilution is needed per well. Each dilution must be measured in duplicate (see section 7.4).

10.3 Working procedure Flow Sheet

Steps	Procedure	Volume/Well	Time/Temperature
1	Pipette 200 µl of standard dilutions, **or** 200 µl of samples into MP wells	200 µl	1 h at +37°C
2	Wash 5 times with washing buffer (solution 6)	5 × 250 µl	5 × 30 s
3	Pipette 200 µl of anti-hGH-DIG, working dilution (solution 2a) into the wells	200 µl	1 h at +37°C
4	Wash 5 times with washing buffer (solution 6)	5 × 250 µl	5 × 30 s
5	Pipette 200 µl of anti-DIG-POD, working dilution (solution 3a) into the wells	200 µl	1 h at +37°C
6	Wash 5 times with washing buffer (solution 6)	5 × 250 µl	5 × 30 s
7	Pipette 200 µl of POD substrate without (solution 4) **or** with substrate enhancer (solution 5) into MP wells	200 µl	10-30 min at +15 to +25°C
8	Measure absorbance at 405 nm (reference wavelength: approx. 490 nm)		

conc. = concentration

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