For Research Use Only

December 3, 2012 Ver. 6

[Rat GH ELISA Kit]

(Code No.:AKRGH-010)

Please, read this instruction carefully before use.

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to Key points for ELISA by movie on our website:
http://www.shibayagi.co.jp/index-E.htm

1. Intended use

Rat GH ELISA Kit is a sandwich ELISA system for quantitative measurement of rat GH (Growth Hormone). This is intended for research use only.

2. Storage and expiration

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Reagents, once opened, should be used as soon as possible to avoid losing its optimal assay performance by storage environment.

3. Introduction

GH (Growth hormone, Somatotrop(h)ic hormone, STH, Somatotropin) is a simple protein mostly produced by acidophilic cells (somatotrophs) of the anterior pituitary gland. Its expression is observed also in the brain and lymphocytes. A similar protein, GH2, is found in human placenta, while rat placental lactogen is similar to prolactin.

GH acts on the liver, muscle, kidney, cartilage, fibroblasts, and thymus epithelial cells, causing IGF-1 production, and IGF-1 induces hypertrophy and proliferation of cells, and enhances protein anabolism in the liver and other tissues, proliferation of cartilage cells and synthesis of chondroitin sulfate, and thymulin release in thymus cells.

GH shows biphasic action on glucose metabolism. GH first shows insulin-like action tentatively, but later induces lipolysis and increase of free fatty acids in the adipocytes, upraise of blood glucose level, suppression of glycolysis, increase of glycogen content in muscle, and lowering o f insulin sensitivity in peripheral tissues.

GH also has prolactin-like actions, i.e. retention of Na, K, Mg, Ca, and P, promotion of Ca absorption in the intestine, and mammary gland growth with induction of milk secretion.

Synthesis and secretion of GH is promoted by GHRH, ghrelin, thyroid hormones, cortisol, and retinoic acid. GH secretion is also enhanced by glucagon, vasopression, 2-deoxy-D-glucose, loading of amino acids like arginine, protein intake, TF5, β-endorphine, L-dopa, and α-adrenergic receptor stimulation.

The physiological situations for induction of GH release are hypoglycemia, stress (caused by fever, injury, hemorrhage, ether, anxiety), starvation, exercise, slow wave sleep, etc.

Suppression of GH release is caused by somatostatin (SRIF), activin, β-adrenergic receptor stimulation, glucose, free fatty acids, corticosteroids, high concentration of IGF-1, and high concentration of GH.

The physiological situations for suppression of GH release are hyperglycemia, high blood fatty acids, REM sleep, etc.

GH secretion is known to be episodic, i.e. blood GH level rapidly increases and decreases with certain intervals. Therefore, GH levels of samples obtained by random bleeding show considerably large variation.

4. Assay principle

In Shibayagi's Rat GH ELISA Kit, standards or samples are incubated in monoclonal anti-GH antibody-coated wells to capture GH. After 2 hours incubation and washing, biotin-labeled anti-GH antibody is added and incubated further for 2 hours to bind captured GH. After washing, HRP (horse radish peroxidase)-labeled avidin is added, and incubated for 30 minutes. After washing, HRP-complex remaining in wells is reacted with a chromogenic substrate (TMB) for 30 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured

spectrophotometrically at 450 nm. The absorbance is nearly proportional to GH concentration. The standard curve is prepared by plotting absorbance against standard GH concentrations. GH concentrations in unknown samples are determined using this standard curve.

5. Precautions

- For professional use only, beginners are advised to use this kit under the guidance of experienced person.
- Wear gloves and laboratory coats when handling assay materials.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- This kit contains components of animal origin. These materials should be handled as potentially infectious.
- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1 M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- The materials must not be pipetted by mouth.
- Residual samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate seal supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. ① (including wind from air conditioner), and humidity less than 30%. ①For airstream, refer to [Assay circumstance] on our web site.

6. Reagents supplied

Components	State	Amount
(A) Antibody-coated 96 well-plate	Use after washing	96 wells/1 plate
(B) Rat standard GH (20 ng/ml) (derived from rat)	Concentrated. Use after dilution	100 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-labeled anti-GH antibody	Concentrated. Use after dilution.	100 µl/1 vial
(E) HRP-avidin conjugate	Concentrated. Use after dilution.	100 µl/1 vial
(F) Chromogenic substrate (TMB) solution	Ready for use.	12 ml/1 bottle
(H) Reaction stopper (1M H_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
(I) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal	—	4 sheets
Instruction Manual	—	1 copy

7. Equipments or supplies required but not supplied

☐Use as a check box

☐Purified water (distilled water)

☐Test tubes for preparation of standard solution series.

☐Glassware for dilution of washing buffer (a graduated cylinder, a bottle)

☐Pipettes (disposable tip type). One should be able to deliver 5-10 µl precisely, and another for 10-100 µl and 100-500 µl.

☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 µl.

☐Paper towel to remove washing buffer remaining in wells.
☐A vortex-type mixer.
☐A shaker for 96 well-plate (600-1200rpm)
☐An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle (refer to our web movie [Washing of microplate]).
☐A 96 well-plate reader (450nm ±10nm, 620nm: 600-650nm)
☐Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

8. Preparation of reagents

◆Bring all reagents of the kit to room temperature (20-25 °C) before use.
◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(B) Rat standard GH (20 ng/ml)]

Make a serial dilution of original standard solution to prepare each standard solution. Example is shown below.

Volume of standard solution	Buffer solution	Concentration (pg/ml)
Original solution : 50 µl	450 µl	2000
2000 pg/ml solution : 200 µl	200 µl	1000
1000 pg/ml solution : 200 µl	200 µl	500
500 pg/ml solution : 200 µl	200 µl	250
250 g/ml solution : 200 µl	200 µl	125
125 pg/ml solution : 200 µl	200 µl	62.5
62.5 pg/ml solution : 200 µl	200 µl	31.3
0 (Blank)	200 µl	0

[(D) Biotin-labeled anti-GH]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

[(E) HRP-avidin conjugate]

Prepare working solution by dilution of (E) with the buffer solution (C) to 1:100.

[(I) Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of deionized water.

【Storage and stability】

[(A) Antibody-coated well-plate]

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[(C) Buffer solution] and [(F) Chromogenic substrate solution]

If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using them as soon as possible to avoid influence by environmental condition.

[(D) Biotin-labeled anti-GH] & [(E) HRP-avidin conjugate]

Unused working solution (already diluted) should be disposed.

[(H) Reaction stopper (1 M H_2SO_4)]

Close the stopper tightly and store at 2-8 ° C. It maintains stability until expiration date.

[(I) Concentrated washing buffer (10x)]

The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date. Dispose any unused diluted buffer.

9. Technical tips

- For manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been

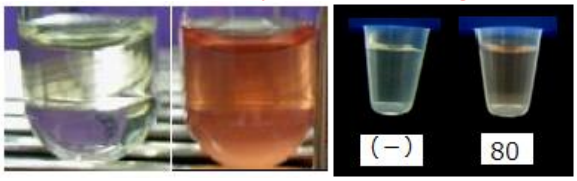
preserved for some period.

- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic substrate (TMB) solution should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the antibody-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

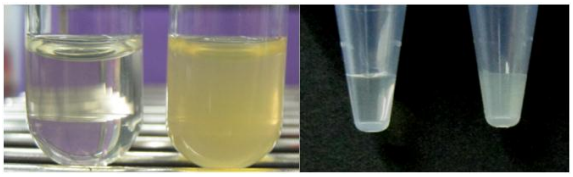
10. Preparation of samples

This kit is intended to measure GH in rat serum (do not use serum-separation-accelerant such as Serum Gel for fear of low assay value) or plasma. The necessary sample volume for the standard procedure is 5 µl.

- When plasma samples are used, we recommend EDTA-2Na (at a final concentration of 1mg/ml) to keep sample' pH and avoid interference of Ca^{2+}.
- Anesthesia while sampling may influence the assay system. We do not recommend ether anesthesia.
- Don' t use sample tubes for human when collecting blood. We have not checked all kinds of sample tubes. Please contact us if necessary.
- Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.
- If presence of interfering substance is suspected, examine by dilution test at more than 2 points.
- Samples should be immediately assayed or stored below –35°C for several days. Defrosted samples should be mixed thoroughly for best results.
- Hemolytic and hyperlipemic serum samples are not suitable.
  *To avoid influence of blood (high lipid or hemolysis, etc.), if your original samples have heavy chyle or hemolysis as the pictures below, do not use them for assay. Abnormal value might be obtained with hemolysis above 80mg/dL with this kit.



Normal | Hemolysis 80mg/dL | normal | Hemolysis 80mg/dL



Normal | Chyle (Highly lipid sample) | Normal | Chyle (Highly lipid sample)

- Sample dilution should be carried out with the buffer solution of the kit using small test tubes such as PP, PE or glass, before assay. Mix well, and pipette 50 µl of diluted sample into a well. In the standard assay procedure, the dilution rate is 10x. You can choose dilution rate 2-10x, if necessary.

Storage and stability

GH in samples will be inactivated if stored at 2-8 °C. If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Avoid repeated freezing and thawing cycles.

11. Assay procedure

Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

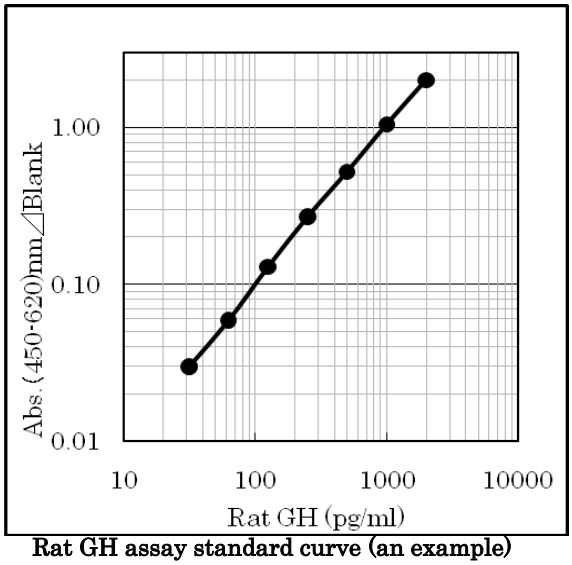
(1) Wash the anti-GH coated plate (A) by filling the well with washing buffer and discard 3 times(*②), then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
(2) Pipette 50 µl of diluted samples to the designated sample wells.
(3) Pipette 50 µl of standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker(*③).
(5) Stick a plate seal (*④) on the plate and incubate for 2 hours at 20-25°C.
(6) Discard the reaction mixture and rinse wells as step (1).
(7) Pipette 50 µl of biotin-labeled anti-GH solution to all wells, and shake as step (4).
(8) Stick a plate seal (*④) on the plate and incubate the plate for 2 hours at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 50 µl of HRP-conjugated avidin solution to all wells, and shake as step (4).
(11) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(12) Discard the reaction mixture and rinse wells as step (1).
(13) Pipette 50 µl of chromogenic substrate solution to wells, and shake as step (4).
(14) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(15) Add 50 µl of the reaction stopper to all wells and shake as step (4).
(16) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

*Refer to the page 7-8 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against GH concentration (pg/ml) on X-axis. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.

(2) Using the standard curve, read the GH concentration of a sample at its absorbance*, and multiply the assay value by dilution factor. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution. * We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.



Rat GH assay standard curve (an example)
Absorbance may change due to assay situation.

13. Performance characteristics

● Assay range

The assay range of the kit is 31.3 ~ 2000 pg/ml. (For 10x dilution, 313 ~ 20000 pg/ml)

● Specificity

The kit uses a monoclonal antibodies specific to rat GH.

*Cross reaction at 2000 pg/ml.

Samples	Cross reaction	Samples	Cross reaction
Rat r-GH	100%	Mouse r-GH	+
Rat Prolactin	0.02%	Mouse TSH	−
Rat Placental lactogen	0.02%	+ : Cross reaction　− : No cross reaction	
Rat TSH	−		
Rat FSH	−		

- Precision of assay

Within assay variation (2 samples, 8 replicates assay,) Mean CV was less than 5 %.

- Reproducibility

Between assay variation (3 samples, 4 days, 4 replicates assay) Mean CV was less than 5 %

- Recovery test

r-GH was added in 3 concentrations to 2 serum samples and was assayed.
The recoveries were 95.1- 106%

- Dilution test

2 serum samples were serially diluted by 3 steps.
The dilution curves showed excellent linearity. (R^2= 0.999)

14. Reference assay data

Rat GH mean assay value: 7.06 ng/ml, SD: 2.17 ng/ml
Strain: CD, male, 6 week-old, serum, fasting, blood collected at 14–15 p.m.
These data should be considered as guidance only. Each laboratory should establish its own normal and pathological reference ranges for GH levels independently.

15. Trouble shooting

- Low absorbance in all wells

Possible explanations:

1) The standard or samples might not be added.
2) Reagents necessary for coloration such as Biotin-labeled antibody, HRP-conjugated avidin, or TMB might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-labeled antibody or HRP-avidin conjugate.
4) Contamination of enzyme inhibitor(s).
5) Influence of the temperature under which the kits had been stored.
6) Excessive hard washing of the well plate.
7) Addition of TMB solution soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

- Intense coloration in all wells including blank

Possible explanations:

1) Improper or inadequate washing. (Change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP-avidin.)
2) Overdeveloping. Incubation time with chromogenic substrate solution should be decreased before addition of reaction stopper.
3) Too high incubation temperature. Adjust the temperature to 20-25°C.

- High coefficient of variation (CV)

Possible explanation:

1) Improper or inadequate washing.
2) Improper mixing of standard or samples.
3) Pipetting at irregular intervals.

- Q-1: Can I divide the plate to use it for the other testing?

A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon

- Q-2: I found there contains liquid in 96 well-plate when I opened the box. What is it?

A-2: When we manufacture 96 well-plate, we put protective solution in wells.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits"** on our website (http://www.shibayagi.co.jp/en/tech_004.html).

Summary of assay procedure ☐ : Use as a check box

***First, read this instruction manual carefully and start your assay after confirmation of details.**

For more details, watch our web movie **[ELISA by MOVIE]** on our website.

☐Bring the well-plate and all reagents to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified watere that returned to 20-25°C.

☐Standard GH solution dilution example:

Concentration (pg/ml)	2000	1000	500	250	125	62.5	31.3	0
Std. GH solution (µl)→	Orig.sol. 50	200*	200*	200*	200*	200*	200*	0
Buffer solution (µl)	450	200	200	200	200	200	200	200

*One rank higher standard.

☐Make the positive control.

			Precautions & related info
☐	Antibody-coated 96 well-plate		
☐	↓Washing 3 times(*②)		*⑥
☐	Diluted Samples / Standards (i.e. buffer 45µl + sample 5µl)	50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 2 hrs at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute Biotin-labeled anti-GH antibody (D) to 100x with buffer (C) returned to 20-25°C.		Dilute reagents during the first reaction.
☐	↓Washing 3 times(*②)		*⑥
☐	Biotin-labeled anti-GH antibody	50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 2 hrs at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute HRP-avidin conjugate (E) to 100x with buffer (C) returned to 20-25°C.		Dilute reagents during the second reaction.
☐	↓Washing 3 times(*②)		*⑥
☐	HRP-conjugated avidin	50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 30 mins at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓Washing 3 times(*②)		*⑥
☐	Chromogenic substrate (TMB)	50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓Shaking(*③), Incubation for 30 mins at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	50 µl	After dispense, the color turns to yellow depending on the concentration.
☐	↓Shaking(*③)		Immediately shake.
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤))		Ref. wave cancels the dirt in the back of plate.

*②After dispensing wash buffer to wells, lightly shake the plate on your palm for 10 sec and remove the buffer. Guideline of washing volume: 300µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP conjugated streptavidin.
Standard of plate-washing pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie **[Washing of microplate]**.

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.

*④Seal the plate during the reaction after shaking. Peel off the protective paper from the seal and stick the seal on the plate. Do not reuse the plate seal used once.
*⑤600-650 nm can be used as reference wavelength.
*⑥After removal of wash buffer, immediately dispense the next reagent.
*⑦Refer to our web movie [Handling of pipetting].
*⑧Refer to our web movie [Assay circumstance].

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	2000 pg/ml	Pos. Control	Sample 8	Sample 16	Sample 24	Sample 32
B	1000 pg/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	500 pg/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	250 pg/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	125 pg/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	62.5 pg/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	31.3 pg/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	0	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

Assay worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

[Storage condition] Store the kit at 2-8°C (Do not freeze).
[Term of validity] 6 months from production (Expiration date is indicated on the container.)

This kit is manufactured by **Shibayagi Co., Ltd.**
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