For Research Use Only

December 3, 2012 Ver. 2

[Rat LH ELISA Kit (S-type)]

(Code No.:AKRLH-010S)

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 LH ELISA Kit (S-type) is a sandwich ELISA system for quantitative measurement of rat LH. 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. Opened reagents should be used as soon as possible to avoid loss of optimal assay performance caused by storage environment.

3. Introduction

LH is produced and stored in basophilic cells called gonadotrophs in the anterior pituitary gland. LH is found in all vertebrates from fishes to mammals. Some reports indicated the presence of LH-like substance in the testis.

LH has a heterodimer structure consisted of α-subunit and β-subunit with a total molecular weight of approximately 29,000. α-Subunit is a glycoprotein which is common to other pituitary hormones follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), while β-subunit is specific to LH, and is also a glycoprotein.

The receptor of LH is a transmembrane receptor of G-protein coupled type, which relates to PKA system and penetrates the cell membrane 7 times.

In females, LH acts on maturated granulosa cells in ovarian follicle, where it causes, in cooperation with FSH, follicular maturation, estrogen production, and ovulation, and after ovulation, acts on corpora lutea and promotes progesterone production and secretion.

In males, LH acts on the interstitial cells (Leidig cells) in the testis, causing production and secretion of androgens, and secondarily promotes spermatogenesis via androgen.

Deficiency of LH leads to the lower secretion of sex steroids, atrophy of interstitial cells, and failure of ovulation and luteinization, while excessive LH causes hyperplasia of testicular interstitial cells followed by atrophy, increased secretion of estrogen or androgen, super-ovulation, and accelerated sexual maturation.

GnRH (LHRH) secreted from hypothalamus act directly on gonadotrophs and stimulate LH secretion. Physiologically, positive feedback of estrogen at preovulatory period causes LH surge via GnRH and leads to ovulation. LH secretion is promoted in postmenopausal period women and in aging in men.

LH secretion was minimized by increased blood levels of sex steroids, opioid peptides especially endorphin, in childhood, and pregnancy.

For details of LH biosynthesis and secretion under various physiological states check our website (English version at http://www.shibayagi.co.jp/en/tech_010_01.html).

4. Assay principle

In Shibayagi's Rat LH ELISA Kit, standards or samples are incubated in monoclonal anti-LH ß antibody-coated wells to capture LH. After 2 hours' incubation and washing, biotin-labeled anti-LH α antibody is added and incubated further for 1 hour to bind with captured LH. After washing, HRP (horse radish peroxidase)-labeled avidin is added, and incubated for 30 minutes. After washing, HRP-complex remaining in wells are reacted with a chromogenic substrate (TMB) for 20 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to LH concentration. The standard curve is prepared by plotting absorbance against standard LH concentrations. LH 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-glasses and protective clothing when handling these reagents.
- The materials must not be pipetted by mouth.
- Unused 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 LH (100ng/ml) (derived from the rat anterior pituitary gland)	Concentrated. Use after dilution	200 µl/1 vial
(C) Assay buffer	Ready for use.	60 ml/1 bottle
(D) Biotin-labeled anti-LH 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
(G) Sample dilution buffer	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

*You can take out 100 µl from vials.

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 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 LH (100ng/ml)]

Make a serial dilution of master standard solutions using Assay buffer (C) to prepare each standard solution.

Volume of standard solution	Assay buffer (C)	Concentration(ng/ml)
Original solution : 50 µl	450 µl	10.0
10 ng/ml solution : 200 µl	200 µl	5.0
5 ng/ml solution : 200 µl	200 µl	2.5
2.5 ng/ml solution : 200 µl	200 µl	1.25
1.25 ng/ml solution : 200 µl	200 µl	0.625
0.625 ng/ml solution : 200 µl	200 µl	0.313
0 (Blank)	200 µl	0

[(D) Biotin-labeled anti-LH antibody]

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

[(E) HRP-avidin conjugate]

Prepare working solution by dilution of (E) with Assay 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 96 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.

[(B) Rat standard LH (100ng/ml)]

Standard solutions prepared above should be used as soon as possible, and should not be stored.

[(C) Assay buffer], [(F) Chromogenic substrate solution] and [(G) Sample dilution buffer]

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-LH antibody] & [(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

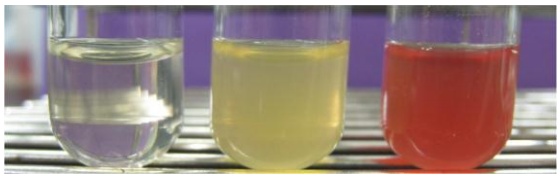
This kit is intended to measure rat serum (do not use serum-separation-accelerant such as Serum Gel for fear of low assay value) and plasma.

The necessary sample volume for the standard procedure is 10 µl.

- **Dilute rat serum (plasma) to 2x using Sample dilution buffer (G). Mix and let it stand still for 10 min at 20-25°C, then further dilute it to 2.5x using Assay buffer (C) (final dilution rate is 5x), and add it to wells for assay. If sample is in good condition, the limit of final dilution rate would be 2.5x. In this case, dilute sample to 2x using Sample dilution bugger (G), mix and let it stand still for 10 min and dilute it further 1.25x using Assay buffer (C).**

  * To avoid influence of blood (high lipid or hemolysis, etc.), 5x at the final dilution is recommended for assay. Even if the final dilution is 5x, abnormal value might be obtained when original sample had heavy chyle or hemolysis as the pictures below.

Normal Chyle hemolysis
(Highly lipid samples) 120mg/dL



Normal Chyle hemolysis
(Highly lipid samples) 120mg/dL

- 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+}. Heparin is not suitable.
- 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.

Storage and stability

Samples should be immediately assayed or stored below –35°C. Defrosted samples should be mixed thoroughly for best results. LH 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 freeze-thaw cycles. Sample dilution should be made just before your assay.

11. Assay procedure

Remove the cover sheet of the 96 well-plate after bringing up to 20-25°C.

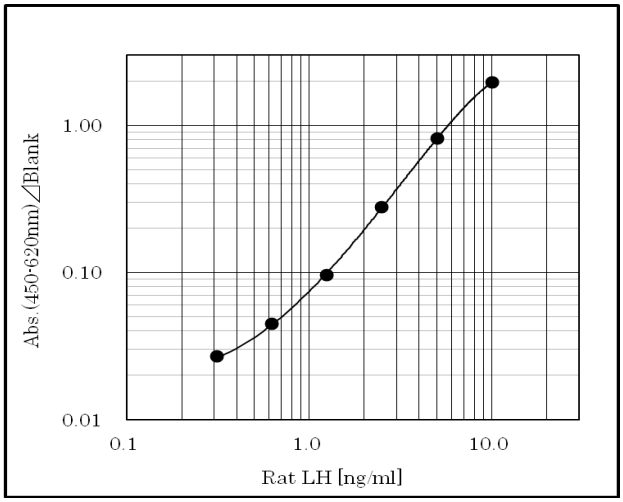
(1) Wash the anti-LH coated plate (A) by filling the well with washing buffer and discard 4 times(*②), then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
(2) Pipette 50µl of properly diluted sample to the designated sample wells. (Limit of dilution is 5x in standard assay procedure)
(3) Pipette 50µl 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 50µl of biotin-labeled anti-LH solution to all wells, and shake as step (4).
(8) Stick a plate seal (*④) on the plate and incubate the plate for 1 hour at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 50µl 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 50µl 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 20 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 LH concentration (ng/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 LH concentration of a sample at its absorbance*, and multiply the assay value by dilution factor if the sample has been diluted. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, repeat the assay after proper dilution of samples with Assay buffer (C).
* 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 LH assay standard curve (an example)
Absorbance may change due to assay situation.
*Absorbance at 450nm minus absorbance at 620nm.

13. Performance characteristics

- Assay range

The assay range of the kit is 0.313 ~ 10ng/ml (for 5x sample dilution, 1.565~50 ng/ml)

- Specificity

The kit uses monoclonal antibodies specific to LH.

- Precision of assay

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

- Reproducibility

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

● Recovery test

Standard LH was added in 3 concentrations to 2 serum samples and was assayed in duplicates.
The recoveries were 95.1 ~106%

● Dilution test

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

14. Reference assay data

Rat LH assay data: Mean 1.93 ng/ml, SD 1.22 ng/ml (Highest: 4.33 ng/ml / Lowest: 0.925 ng/ml)
Subspecies: CD, female, n=9, 8 weeks-old, Serum, fed ad libitum
These are reference data. Blood LH levels may change due to breeding, sampling, and sample storage conditions.

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 4 times to 5-8 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 insert preservation stabilizer 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).

16. References

1) Neonatal immune challenge alters reproductive development in the female rat
L Sominsky et al.
Horm Behav. 2012 Feb 14, in Press.

2) Gonadotropin-Inhibitory Hormone Inhibits GnRH-Induced Gonadotropin Subunit Gene Transcriptions by Inhibiting AC/cAMP/PKA-Dependent ERK Pathway in L β T2 Cells.
Son YL et al.
Endocrinology. 2012 May;153(5):2332-43.

For more information, refer to [User's Publication] on our website.

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 water that returned to 20-25°C.

☐Standard LH solution dilution example: Use Assay dilution buffer (C) returned to 20-25°C.

Concentration (ng/ml)	10	5.0	2.5	1.25	0.625	0.313	0
Std. LH solution (µl)→ Ori.Sol.	50	200	200	200	200	200	0
Buffer solution (µl)	450	200	200	200	200	200	200

*One rank higher standard.

☐Sample dilution-1: Dilute rat sample to 2x using Sample dilution buffer (G), mix and let it stand still for 10 minutes at 20-25°C.

☐Sample dilution-2: Dilute the above prepared sample to 2.5x using Assay buffer (C) returned to 20-25°C.

			Precautions & related info
☐	Antibody-coated 96 well-plate		
☐	↓ Washing 4 times(*②)		*⑥
☐	Properly diluted Samples /Standards	50 µl	***⑦ [Handling of pipetting]***
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))		***⑧ [Assay circumstance]***
☐	Dilute Biotin-labeled anti-LH antibody (D) to 100x with Assay buffer (C) returned to 20-25°C.		Dilute reagents during the first reaction.
☐	↓ Washing 4 times(*②)		*⑥
☐	Biotin-labeled anti-LH antibody	50 µl	***⑦ [Handling of pipetting]***
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		***⑧ [Assay circumstance]***
☐	Dilute HRP-avidin conjugate (E) to 100x with Assay buffer (C) returned to 20-25°C.		Dilute reagents during the second reaction.
☐	↓ Washing 4 times(*②)		*⑥
☐	HRP-conjugated avidin	50 µl	***⑦ [Handling of pipetting]***
☐	↓ Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))		***⑧ [Assay circumstance]***
☐	↓ Washing 4 times(*②)		*⑥
☐	Chromogenic substrate (TMB)	50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 20 minutes 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 4 times to 5-8 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	10 ng/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
B	5 ng/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
C	2.5 ng/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
D	1.25 ng/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
E	0.625 ng/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
F	0.313 ng/ml	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
G	0	Sample 8	Sample 16	Sample 24	Sample32	Sample 40
H	Sample 1	Sample 9	Sample 17	Sample 25	Sample33	Sample 41

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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