For Research Use Only August 2, 2016 Ver. 1

[Rat TSH ELISA Kit (SII-type)]

(Code No.:AKRTS-010S2)

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: www.shibayagi.co.jp/index-E.htm

◆ Technical tips which should be checked before use

- Anesthesia such as deep ether anesthesia or stress at blood sampling may influence on TSH level. We recommend barbiturates as anesthesia, though they also influence hypothalamus to minimize TRH release (Endocrinology 99: 875-880, 1975).
- To stabilize sample pH and avoid the effect of calcium ion, we recommend EDTA-2Na at final concentration of 1mg/ml as anticoagulant. For other anticoagulants, please ask us. Especially in case of high heparin concentration, the assay value may lower or may not be detected.
- When using serum separating tubes for human, the technicians may need to check the influence to the assay in advance.
- Samples should be immediately assayed or stored below –35°C. Defrosted samples should be mixed thoroughly for best results. Avoid repeated freeze-thaw cycles.
- Samples should be used for assay as soon as possible after collection.
- If sample is turbid or contains insoluble materials, centrifuge and use clear supernatant fluid.
- Organic solvents may influence assay results.
- High hemolysis may influence assay results.
- If presence of interfering substances is suspected, examine by dilution test at more than 2 points.
- Sample dilution should be carried out beforehand in sample tubes made of PP, PE or glass.
- 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.
- The chromogenic substrate (TMB) solution should be almost colorless or clear pale blue before use. Avoid direct sunlight during storage.
- Make sure to stick a plate seal to a plate at each incubation time in order to avoid dryness of well, contamination of foreign substances, temperature fluctuation and evaporation of aliquot reagents.
- 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.
- However, if you cannot avoid the situation above, make sure to put a plate seal and try the followings:
  Incubation using an incubator; in a styrofoam box; or on a tube lack. For more information, watch our web movie [Assay circumstance].

1. Intended use

Rat TSH ELISA Kit (SII-type) is a sandwich ELISA system for quantitative measurement of rat TSH (Thyroid-stimulating hormone), and 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 less than optimal assay performance caused by storage environment.

3. Introduction

TSH (Thyroid-stimulating hormone, Thyrotropin) is a glycoprotein hormone of 28kDa consisting of α- and β-subunits. The former is common to other pituitary glycoproteins, LH and FSH, while the latter has specific structure to TSH. TSH shows microheterogeneity due to the difference in sugar chain structure. TSH is produced and secreted by basophilic cells called thyrotrophs in the anterior pituitary throughout vertebrates. TSH enhances thyroid hormone synthesis and release by increasing inorganic iodide uptake and iodination of thyroglobulin in the thyroid gland, stimulates glucose utilization and degradation of lipid, and also causes exophthalmos. TSH secretion is promoted directly by TRH (thyrotropin-releasing hormone) and also indirectly by estrogen and insulin. Cold exposure stress increases blood TSH level. TSH secretion is lowered by somatostatin, thyroid hormones, GH, glucocorticoids, and β-endorphin, and also by stress and starvation. Blood TSH level shows diurnal variation.

4. Assay principle

In Shibayagi's Rat TSH ELISA Kit (SII-type), standards or diluted samples are incubated in anti-TSH antibody-coated wells to capture TSH. After 2 hours' incubation and washing, biotin-labeled anti-TSH antibody is added and incubated further for 1 hour to bind with captured TSH. 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 proportional to TSH concentration. The standard curve is prepared by plotting absorbance against standard concentrations. TSH 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 clean laboratory glassware, 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.
- 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.
- 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.

6. Reagents supplied

Components	State	Amount
(A) Anti-TSH-coated plate	Use after washing	96 wells/1 plate
(B) Standard TSH solutions (derived from rat) (6 concentrations, ng/ml: color) ①18.0:● ②7.20:● ③2.88:● ④1.15:● ⑤0.460:● ⑥0.184:○	Ready for use.	450 µl/1 vial/conc. (6 conc., 6 vials)
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-conjugated anti-TSH antibody	Concentrated. Use after dilution.	100 µl/1 bottle
(E) HRP-conjugated avidin	Concentrated. Use after dilution.	100 µl/1 bottle
(F) Substrate chromogen reagent (TMB)	Ready for use.	12 ml/1 bottle
(H) Reaction stopper (1M H_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
(I) Washing buffer concentrate (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal / Plate cover	—	4 sheets / 1 cover
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 10-100 µl precisely, and another for 50-500 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 washing 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 20-25 °C for about 2 hours before use.
◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(D) Biotin-conjugated anti-TSH antibody]
You can take out 100µl of (D). Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

[(E) HRP-conjugated avidin]
You can take out 100µl of (E). Prepare working solution by dilution of (E) with the buffer solution (C) to 1:100.

[(I) Washing buffer concentrate (10x)]
Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with purified (distilled) water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of purified (distilled) water.

【Storage and stability】

[(A) Anti-TSH-coated 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) Standard TSH solutions]

In case of partial use, take out the standard solutions from refrigerator just before assay. The rest of the standards will be stable until expiration date when tightly capped and immediately stored at 2-8 °C.

[(C) Buffer solution] and [(F) Substrate chromogen reagent]

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-conjugated anti-TSH antibody] & [(E) HRP-conjugated avidin]

Unused working solution (already diluted) should be disposed. If you use only a part of the reagents, the rest of them is stable until expiration date, if stored tightly closed at 2-8 °C.

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

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

[(I) Washing buffer concentrate (10x)]

The rest of undiluted buffer will be stable until expiration date when tightly capped and stored at 2-8 °C.

Dispose any unused diluted washing buffer.

9. Preparation of assay samples

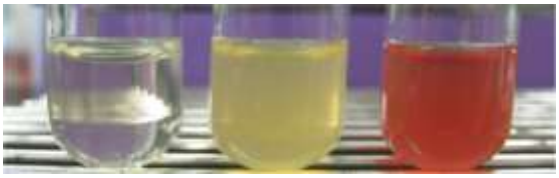
This kit is principally intended to measure TSH in rat serum and plasma.

- Sample dilution should be carried out with the buffer solution of the kit using small test tubes before assay. Mix well and pipette diluted sample into wells for assay. In the standard assay procedure, the dilution rate is 5.0x.

  *Regarding sample dilution with buffer: after dilution with the buffer, mix well to blend buffer and sample. If you have a rolling mixer, use it to mix for about 15 minutes. If you don't have it, let sample stay still for 15 minutes and vortex it before dispensing to wells.
- **Avoid using hemolytic or high lipid samples as they may cause abnormal value.**

  *Even with the final dilution rate of 5.0x, if chyle or hemolysis in your original samples show higher level than the followings, avoid using them for assay because abnormal value may be obtained.

Normal Chyle (Highly lipid sample) hemolysis 440mg/dL



Normal Chyle (Highly lipid sample) hemolysis 440mg/dL

- To stabilize sample pH and avoid the effect of calcium ion, we recommend EDTA-2Na at final concentration of 1mg/ml as anticoagulant. For other anticoagulants, please ask us. Especially in case of high heparin concentration, the assay value may lower or may not be detected.

Stability and storage of samples

If you would like to store samples for a long period, tightly close the container and store below –35°C. Avoid repeated freezing and thawing. Samples should be diluted just before assay.

10. Assay procedure

Reagents to be diluted next should be prepared before starting washing procedure.
Remove the cover sheet of the 96 well-plate after bringing up to 20-25°C.

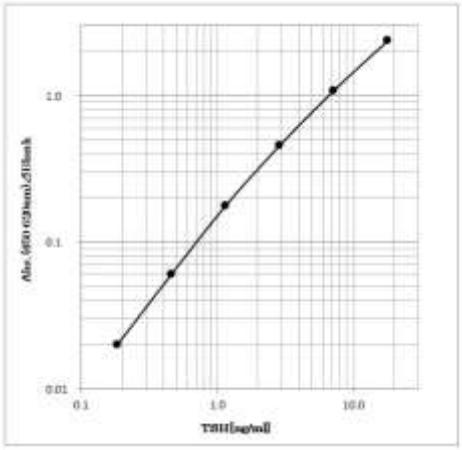
(1) Wash the anti-TSH coated plate (A) by filling the wells with washing buffer and discard 4 times (*②), then strike the plate upside-down onto several layers of paper towels to remove residual buffer drops in the wells.
(2) Pipette 100µl of diluted sample to the designated sample wells (dilution rate 5.0x in the standard procedure).
(3) Pipette 100µl of each standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker (*③).
(5) Stick a plate seal on the plate and incubate for 2 hours at 20~25°C.
(6) Discard the reaction mixture in the wells and rinse wells as step (1).

(7) Pipette 100µl of Biotin-conjugated anti-TSH antibody, which is diluted to 100x by the buffer, to each well, and shake the plate gently on a plate shaker (*③).
(8) Stick a plate seal on the plate and incubate for 1 hour at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 100µl of HRP-conjugated avidin, which is diluted to 100x by the buffer, to each well, and shake the plate gently on a plate shaker (*③).
(11) Stick a plate seal on the plate and incubate for 30 minutes at 20-25°C.
(12) Discard the reaction mixture and rinse wells as step (1).
(13) Pipette 100µl of substrate chromogen reagent to wells and shake the plate gently on a plate shaker (*③).
(14) Stick a plate seal on the plate and incubate the plate for 30 minutes at 20-25°C.
(15) Add 100µl of the reaction stopper to all wells and shake the plate gently on a plate shaker (*③).
(16) After shaking, measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a microplate reader within 30 minutes. 600~650nm is allowed for reference wavelength .

*Refer to the page 7 for notes of *② and *③.

11. Calculations

TSH assay standard curve (an example below)
Absorbance may change due to assay environment.



(1) Prepare a standard curve in every assay by plotting absorbance* (Y-axis) against TSH concentration (ng/ml) on X-axis. *Absorbance at 450nm minus absorbance at 620nm.
(2) Using the standard curve, read TSH concentration of samples at their absorbance, and multiply the assay value by dilution rate. 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.
(3) We recommend the use of 3rd order regression curve for log-log plot, or 4/5 parameters method for log-normal plot in computer calculation. We are providing a convenient assay calculation template for EXCEL on our website.
(4) Physiological or pathological situation of animals should be judged comprehensively taking other examination data into consideration.

12. Performance characteristics

- Assay range

The kit's standard curve range is 0.184~18.0 ng/ml. (for 5x sample dilution, 0.920~90.0 ng/ml)

- Specificity

Monoclonal antibodies specific to rat TSH are used in this kit.
Reactivity for closely related substances to TSH is shown below.

Substance	Cross reactivity	Substance	Cross reactivity
Rat TSH	100%	Rat FSH	No cross reaction
Rat LH	No cross reaction	Rat GH	No cross reaction

TSH is a heterodimer consisting of α- and β-subunits. Some RIA assays based on competitive binding recognize only one epitope, and have the possibility of measuring free β-subunit. Shibayagi's TSH ELISA kit uses two antibodies, and each of them recognizes α-subunit and β-subunit, respectively. So, the assay system is specific to native TSH. Thereofre, the TSH assay values after thyroidectomy or goitorogen administration may be different from the data reported with RIA measurement. Ref. Mori M, Oshima K, et al. Acta Endocrinol, 105: 49-56, 1984

- Precision of assay

Within assay variation (5 samples, 8 replicates assay,) Mean CV is less than 10 %.

- Reproducibility

Between assay variation (2 samples, 4 days, assayed in 4 replicates) Mean CV is less than 10 %

- Recovery test

Standard TSH was added in 5 concentrations to serum samples and assayed; the recoveries were 98.3 ~101%.

- Dilution test

Three serum samples were serially diluted by 3 steps.
The dilution curves showed excellent linearity with R^2 of 0.999.

13. Reference assay data

Rat TSH assay data: 3.26~4.65 ng/ml; subspecies: CD(SD); male; n=5; 9 weeks-old of age; fasted.
Rat TSH assay data: 2.50~4.04 ng/ml; subspecies: CD(SD); male; n=5; 7 months-old of age; non-fasted.
Rat TSH assay data: 8.09~11.9 ng/ml; subspecies: CD(SD); male; n=5; 9 weeks-old of age; non-fasted; thyroidectomized.

*These are reference data. Blood TSH levels show diurnal change, and may change due to breeding, sampling, and sample storage conditions.

14. 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 taken out from a refrigerator might cause poor coloration owing to low temperature.

- The OD of blank is higher than that of the lowest standard concentration (0.184 ng/ml)

Possible explanations: Improper or inadequate washing. (Change washing repetition from 4 times to 5-8 times after the reaction with HRP-avidin.)

- 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).

Summary of assay procedure [Standard 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 back all the necessary reagents to 20-25°C for 2 hours.

☐ Washing buffer must be diluted to 10x with purified water of 20-25°C.

☐ Preparation of positive sample

			Precautions & related info
☐	Anti-TSH-coated plate		
☐	↓ Washing 4 times(*②)		*⑥
☐	Properly diluted samples and standard TSH solutions	100 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute Biotin-conjugated anti-TSH antibody (D) to 100x with Buffer solution (C) returned to 20-25°C.		Dilute reagents during the first reaction.
☐	↓ Washing 4 times(*②)		*⑥
☐	Biotin-conjugated anti-TSH antibody	100 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute HRP-conjugated avidin (E) to 100x with Buffer solution (C) returned to 20-25°C.		Dilute reagents during the second reaction.
☐	↓ Washing 4 times(*②)		*⑥
☐	HRP-conjugated avidin	100 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)	100 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	100 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. Be careful with dryness of wells and add the next reagent immediately. In case of adding wash buffer by a pipette, recommended volume of wash buffer is 300µl/well. One of the solutions when blank OD is higher than that of the lowest standard is to increase washing times up to 5-8 times instead of the standard 4 times with the same flow rate after the reaction with HRP-conjugated avidin.
Standard plate-washing pressure: 5-25 ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1,200 rpm 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].

An example of work sheet

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	**18.0 ng/ml**	Sample2	Sample10	Sample 18	Sample26	Sample34
B	**7.20 ng/ml**	Sample3	Sample11	Sample19	Sample27	Sample35
C	**2.88 ng/ml**	Sample4	Sample12	Sample20	Sample28	Sample36
D	**1.15 ng/ml**	Sample5	Sample13	Sample21	Sample29	Sample37
E	**0.460 ng/ml**	Sample6	Sample14	Sample 22	Sample30	Sample38
F	**0.184 ng/ml**	Sample7	Sample15	Sample23	Sample31	Sample39
G	**0 (blank)**	Sample8	Sample16	Sample24	Sample32	Sample40
H	Sample 1	Sample9	Sample17	Sample25	Sample33	Sample41

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 in dark place (Do not freeze).
[Term of validity] 6 month from production (Expiration date is indicate upon the container)

This kit is manufactured by shibayagi Co., Ltd.
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