SHIBAYAGI	Rat TSH ELISA KIT	Research Reagent

Please, read this instruction carefully before use.

This is an ELISA (Enzyme Linked ImmunoSorbent Assay) kit for measurement of rat TSH (thyroid-stimulating hormone) with high sensitivity using Sandwich assay principle.

It is also possible to measure mouse TSH with this kit. The supporting data are shown in the last part of this instruction.

[Advantage]

(1) Rapid assay (total reaction time: 3 hours 50min.).
(2) A small sample volume (10µl in the standard procedure).
(3) An ecologically excellent preservative is used.
(4) Every reagent is provided in liquid form and ready to use.
(5) Excellent precision and reproducibility.

[Components]

	Reagents	Amounts
(A)	Anti-TSH-coated plate	96 wells(8x12) / 1 plate
(B)	Standard rat TSH solution (360ng/ml, NIDDK rat TSH RP-3 equivalent)	200µl / 1 vial
(C)	Buffer solution	60ml/ 1 vial
(D)	Biotin-conjugated anti-TSH antibody	50µl/ 1 vial
(E)	Peroxidase-conjugated streptavidin	50µl/ 1 vial
(F)	Chromogenic substrate reagent (TMB)	12ml/ 1 vial
(H)	Reaction stopper (1M H_2SO_4)	12ml/ 1 vial
(I)	Concentrated washing buffer(10x)	100ml/ 1 bottle

[Assay sample]

Rat or mouse serum or plasma : 10µl/well in the standard procedure. (Serum is preferable.)
Please, use EDTA at the final concentration of 1mg/ml for obtaining plasma.
The volume of assay sample can be applied in the range of 10 ~ 50µl. In such case the final volume of the liquid in the well should be adjusted to 50µl using assay buffer (C).
It would be also convenient to dilute the assay samples first in test tubes, and pipette 50µl of the diluted sample to a well.

[Assay range]

0.288~36ng/ml

[Assay operation]

1. Equipments necessary but not included in the kit.

(1) Micropipette (a micropipette able to deliver sample volume with high precision.), and a pipette for repetitive dispensing.
(2) Microplate washing apparatus (a microplate washer or a flashing bottle with a nozzle).
(3) A microplate reader (A densitometer for microplate).

2. Preparation of reagents

(1) Washing buffer: Dilute the concentrated washing buffer (I) to 10X with purified water.
(2) Biotin-conjugated anti-TSH (D) : Dilute to 200X with the buffer solution(C).
(3) Peroxidase-streptavidin solution (E): Dilute to 200X with the buffer solution(C).
(4) Other reagents are used as they are.
(5) All the reagent solutions should be used after getting back to room temperature (20-25C).

3. Preparation of standard solutions

(An example) Dilute the original standard solution (B) with the buffer solution to prepare 36ng/ml, then prepare lower standard solutions by a dilution program shown below.
(You can use other mode of dilution for a set of standard solutions.)

Concentration. (ng/ml)	36	18	7.2	3.6	1.44	0.72	0.288	0
Std. Sol. (μl)	Orig.sol. 50	200*	200*	200*	200*	200*	200*	0
Buffer (μl)	450	200	300	200	300	200	300	200

*One rank higher standard solution

4. Assay procedure

(1) Remove the cover sheet of the microplate (A) after getting back to room temperature.
(2) Rinse the anti-TSH coated wells (A) by filling the washing buffer and discard 4 times, then strike the plate upside-down onto folded several sheets of paper towel, and remove the excess buffer.
(3) Pipette 40μl of buffer solution into the wells for samples, then add 10μl of sample.
Alternatively, if you use larger sample volumes (x μl), the volumes of buffer(C) should be (50 − x) μl to adjust the final volume to 50 μl.
It would be also convenient to dilute the assay samples first in test tubes, and pipette 50μl of the diluted sample to a well.
(4) Pipette 50μl of the standard solution to the wells for preparing a standard curve.
(5) Shake the plate gently on a plate shaker.
(6) Incubate for 2 hours at room temperature (20-25C).
(7) Discard the reaction mixture, and then wash the plate 4 times as described in (2), and remove excess washing buffer remaining in the wells as (2).
(8) Pipette 50μl of biotin-conjugated anti-TSH solution to all wells. Then shake gently on a plate shaker.
(9) Incubate the plate for 1 hour at room temperature.
(10) Discard the reaction mixture, and then wash the plate 4 times as (2), and remove excess washing buffer

(11) Pipette 50µl of HRP-conjugated avidin solution to all wells, and shake as (5).
(12) Incubate for 30 minute at room temperature.
(13) Discard the reaction mixture, and wash the plate as (2), and remove excess washing buffer
(14) Pipette 50µl of chromogenic substrate solution to wells, and shake as (5).
(15) Let the plate stand for 20 minutes at room temperature.
(16) Add 50 µl of the reaction stopper (H) to all wells and shake.
(17) Measure the absorbance of each well at 450 nm (sub-wave length, 620nm) by a plate reader within 30 minutes.

[Summary of Assay Procedure]

Antibody-coated 96 well plate
↓
Washing 4times
↓
Sample* or Standard 50µl
↓
Shaking and reaction for 2 hours at 20~25C
↓
Washing 4 times
↓
Biotin-conjugated anti-TSH 50µl
↓
Shaking and reaction for 1 hour at 20~25C
↓
Washing 4 times
↓
Peroxidase-conjugated avidin 50µl
↓
Shaking, and reaction for 30min at 20~25C
↓
Washing 4 times
↓
Chromogenic substrate solution 50µl
↓
Shaking, and reaction for 20 mins. at 20~25C
↓
Reaction stopper 1M H_2SO_4 50µl
↓
Shaking and measurement of absorbance
at 450nm(sub. 620nm)

*Refer to the detailed procedure (3) for sample volume.

[Calculation of TSH concentration]

(1) Prepare a standard curve using normal or semi-logarithmic or bi-logarithmic section paper by plotting absorbance* (Y-axis) against standard concentration (ng/ml) on X-axis. For the manual reading from the standard curve, we recommend the use of bi-logarithmic section paper.

*Absorbance at 450nm minus absorbance at 620nm.

(2) Read TSH concentration of a sample from its absorbance*, and multiply the assay value by dilution rate (in the standard procedure, the dilution rate is 5) . Though the assay range is wide enough, in case the absorbance of some samples are 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 or 4 parameter method in computer calculation. If you use logarithm transformation for TSH concentration and absorbance, the fitness of the 3rd order regression will be improved.

[Important notice in the treatments]

1. Treatment of assay samples

(1) Use serum samples or plasma samples obtained by indicated method.

(2) Turbid samples or those containing insoluble materials should be centrifuged before assay and remove those materials.

(3) Measure the samples as soon as possible after sampling.

(4) It would be also convenient to dilute the assay samples first in test tubes, and pipette 50µl of the diluted sample to a well.

2. Storage of assay samples.

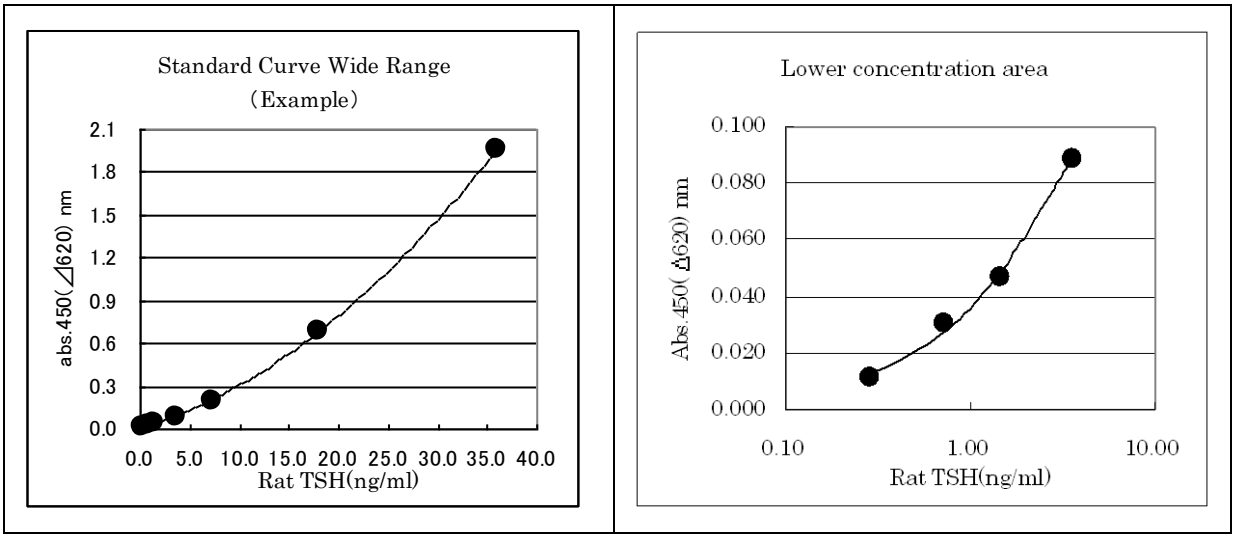
If assay samples have to be stored for a long period, freeze samples and store below –35C. Avoid repeated freezing and thawing.

3. Influence of interfering substances

If presence of interfering substances is suspected, examine by a dilution test using more than 2 points.

[Assay range and assay validation]

1. A model standard curve



2. Specificity

Specificity by dot blot test

Dot sample	Capture antibody	Labeled antibody
Rat TSH	+++	+++
Rat LH/FSH/CG	−	++
Mouse TSH	+++	+++
Mouse LH/FSH/CG	−	++
Human TSH	++	++

Cross-reactivity of the assay system

Substance added	Reactiviry (found)
Rat TSH	100%
Rat LH	ND
Rat FSH	ND
Rat GH	ND
Mouse TSH	100%
Mouse LH	ND
Mouse FSH	ND
Mouse GH	ND
Human TSH (*)	85%

Substances were added at : 100ng/ml

ND: not detected (less than lowest assay limit)

*:Acris Antibodies GmbH/PA1199

3. Precision

Within assay variation

	Samples		
Wells	A	B	C
1	21.4	8.51	1.62
2	21.4	8.81	1.75
3	21.5	8.85	1.83
4	21.9	8.74	1.94
5	22.0	8.69	1.75
mean	21.6	8.72	1.78
SD	0.29	0.13	0.12
CV(%)	1.35	1.52	6.54

Unit: ng/ml

4. Reproducibility

Between assay variation

	Assays						
Samples	Day 1	Day 2	Day 3	Day 4	mean	SD	CV (%)

E	23.7	23.3	23.5	23.6	23.5	0.18	0.77
F	6.08	5.89	5.94	6.00	5.98	0.08	1.35
G	1.33	1.31	1.41	1.39	1.36	0.05	3.58

Unit: ng/ml, n=4

5. Recovery test

Sample D

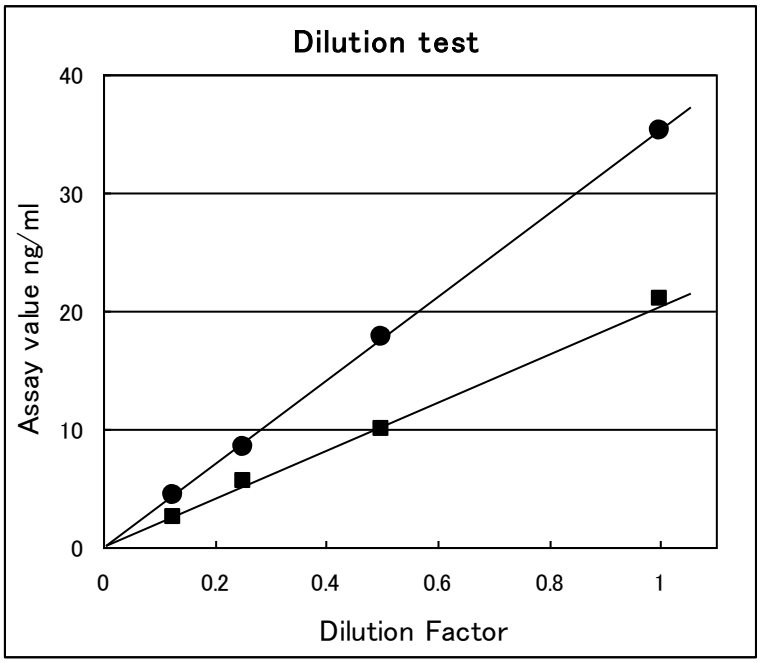
Added	Found	Recovered	Recovery (%)
0.00	15.2	-	-
5.97	21.3	6.10	102
14.9	29.9	14.7	99.0
18.0	32.5	17.3	96.1

Sample E

Added	Found	Recovered	Recovery (%)
0.00	4.44	-	-
2.45	6.93	2.49	102
3.32	7.85	3.41	103
4.76	9.58	5.14	108
5.41	9.89	5.45	101

Unit: ng/ml, n=2

6. Dilution test



[Assay values of TSH in normal rats]

Rat: SD rats, males from Charles River Laboratories Japan

Samples: Sera obtained 14:00~16:00

Sample No,	Age (weeks)	TSH (ng/ml)
1	4	0.522
2	4	1.75
3	4	3.60
4	4	1.30
5	4	7.25
6	4	4.20
7	4	2.77
8	4	6.07
	Mean	3.43
	SD	2.34

n=2

Basic data for mouse serum TSH measurement

Mouse TSH is expressed in terms of NIDDK-rat TSH RP-3 equivalent.

[Precision]

Well＼Sample	A	B
1	13.8	2.23
2	14.7	2.2
3	14	2.2
4	14.1	2.21
5	13.8	2.22
Mean	14.08	2.212
SD	0.370135	0.013038
CV(%)	2.62	0.59

[Reproducibility]

Assay＼sample	E	F	G
Day 1	18.1	3.59	0.71
Day 2	18.2	3.59	0.7
Day 3	18	3.59	0.69
Day 4	18	3.57	0.69
Mean	18.075	3.585	0.6975
SD	0.095743	0.01	0.009574
CV(%)	0.53	0.28	1.37

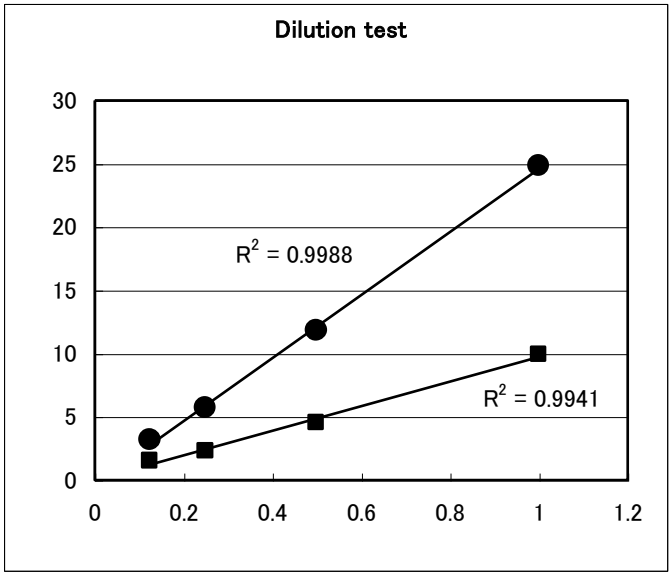
Unit : ng/ml, n=4

[Recovery test]

Sample C			
Added	Found	Recovered	Recovery (%)
0	0.88		
1.58	2.39	1.52	95.6
1.98	2.66	1.79	90.2
2.38	3.09	2.21	93.1
Sample D			
Added	Found	Recovered	Recovery (%)
0	9.65		
17.3	25.8	16.1	93.4
21.6	31.1	21.4	99.2
25.9	34.1	24.5	94.4

Unit : ng/ml, n=2

[Dilution test]



n=2

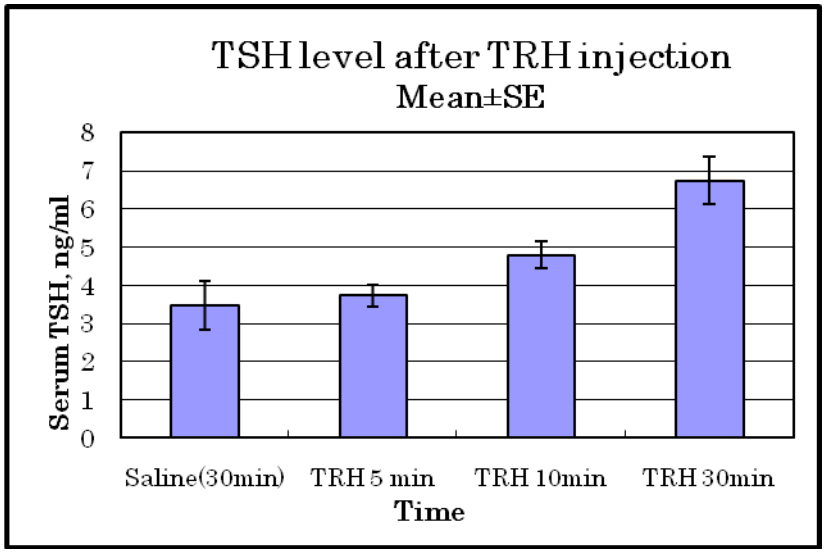
[TRH administration experiment]

Mouse: ICR mouse(Japan Charles-River), 6 weeks old males

TRH :20μg in 400μl/head, i.v. injection. Serum samples

	Physiol. saline	TRH		
Animal No.	Cont.	5min	10min	30min
1	2.44 ng/ml	4.19	6.02	6.82
2	1.45	2.71	3.88	6.58
3	4.43	4.06	4.75	4.96
4	4.42	3.94	3.79	8.95
5	4.59	4.55	4.86	8.47
6		2.73	5.41	6.85

7		3.86		4.5
Mean	3.47 ng/ml	3.72	4.785	6.732857
SD	1.43350	0.71805	0.86391	1.63843
SE	0.64108	0.27139	0.35269	0.61927



[Statements and precaution]

(1) The reagents included in this assay kit should be used only for research works.

(2) The reagent solutions of the kit should be used principally immediately after reconstitution. Otherwise, keep them in a dark place with the temperature 2-8C, and use them within 3 days.

(3) The reagents were prepared to give accurate results by their combination within the kit. So, do not combine the reagents in the kit of other lot number. Even the lot number is the same, do not mix the reagents with those that have been preserved for some period.

(4) Pipetting and dilution of the reagent solutions should be made accurately because these steps influence the assay precision.

(5) Do not dry the assay plate to avoid denaturation of the coated antibody.

(6) Measurement of the reaction time should be started from the pipetting of reagent to the first well.

(7) Prepare the standard curve in each assay.

(8) Dilution of the assay sample must be carried out using the buffer solution attached to the kit.

(9) Storage condition for the kit should be strictly followed.

(10) Be careful not to allow the reagent solutions of the kit to touch the skin and mucus. Especially be careful for the stopping solution because it is 1M sulfuric acid.

(11) HRP-conjugated reagent solution, chromogenic substrate solution, and reaction stopper must be avoided from contacting with any metal.

(12) In treating assay samples of animal origin, be careful for possible biohazards.

(13) As the antibody-coated plate is module type of 8wells x 12 rows, each row can be separated by a cutter and used independently.

[Storage condition]

Store the kit at 2~8C.　Do not freeze.

[Term of validity]

Six months from production.　Expiration date is indicated on the container.

[Unit of package]

96-wells/1 plate

[Product code]

AKRTS-010

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