For in vitro laboratory use only

March 3, 2010 Ver. 3

[Rat IgE ELISA Kit]

(Code No.:AKRIE-011)

Please, read this instruction carefully before use.

Contents

This kit is manufactured by Shibayagi Co., Ltd.

Use only the current version of Instruction Manual enclosed with the kit!

1. Intended use

Rat IgE ELISA Kit is a sandwich ELISA system for quantitative measurement of rat IgE.

Features

(1) This is intended for research use only.

(2) Rapid assay (total reaction time: 3 h 20min.).

(3) This kit is for IgE in rat serum or plasma.

(4) A small sample volume of serum or plasma (5 μl) is needed.

(5) Assay format is 96 wells.

(6) Standard IgE is derived from rat.

(7) All reagents are provided in liquid form.

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 in optimal assay performance caused by storage environment.

3. Introduction

IgE (Immunoglobulin E) is a glycoprotein with a molecular size of 190kDa composed of 2 light chains and 2 heave chains (Hε). In electrophoresis, it moves to γ1 region. Its biological half life is about 3 days, and its blood level in

normal human subject is very low, about 300ng/ml. The blood level of IgE increases markedly in parasite infection and in hay fever. IgE that is responsible for allergy has been called "reagin". Sensitization by an allergen increases reagin IgE which binds to Fc ε R1 receptor in basophilic leucocytes and mast cells at Fc region and sensitizes those cells. If the allergen binds the sensitized cells, they will be degranulated and release histamin, serotonin, protease, heparin, chemotactic factor, prostaglandins, leucotriens, and so on, causing bronchoconstriction, mucous edema, and hypersecretion, and leads to type I allergic reactions like bronchial asthma, hives, allergic rhinitis, anaphylaxis, and so on. This kit is for measurement of total rat IgE. Shibayagi also provides ELISA kits for total and allergen(OVA, DNP)-specific IgE of mouse.

4. Assay principle

In Shibayagi's Rat IgE ELISA Kit, standards or samples and biotin-conjugated anti-IgE antibody are incubated in monoclonal antibody-coated wells to capture IgE bound with biotin-conjugated anti IgE antibody. After 2 hours' incubation and washing, HRP (horse radish peroxidase) conjugated avidin is added, and incubated for 1 hour. After washing, bound HRP-conjugated avidin is reacted with a chromogenic substrate reagent (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 nearly proportional to IgE concentration. The standard curve is prepared by plotting absorbance against standard IgE concentrations. IgE 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.
- 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 reagent containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection 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 cover supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25℃ strictly. Avoid airstream velocity over 0.4 m/sec. (including wind from air conditioner), and humidity less than 30%.

6. Technical tips

- In 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. Otherwise, store them in a dark place at 2-8 °C.
- 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 sucstrate reagent (TMB) 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 of over 0.4 m/sec. and the humidity of less than 30%, completely close each well in addition to cover the well plate with a plate cover in each step of incubation.
  Ex.) Cover the well plate with parafilm, and put the plate cover on it. Or place the well plate with the plate cover in an incubator, or in a styrofoam box. Take the best way depending on situation of each laboratory.

7. Reagents supplied

Components	State	Amount
(A) Antibody-coated plate	Use after washing	96 wells/1 plate
(B) IgE Standard solution (100ng/ml)	Concentrated. Use after dilution	600 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-conjugated anti-IgE antibody	Concentrated. Use after dilution.	100 µl/1 vial
(E) HRP-conjugated avidin	Concentrated. Use after dilution.	200 µl/1 vial
(F) Chromogenic substrate reagent (TMB)	Ready for use.	12 ml/1 bottle
(H) Reaction stopper (1M H_2SO_4)	Ready for use.	12 ml/1 bottle
(I) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate cover	−	1 plate
Instruction Manual	−	1 copy

8. Equipments or supplies required but not supplied

- 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 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 or 100 µl.
- Paper towel to remove washing buffer remaining in wells.
- A vortex-type mixer.
- A shaker for 96 well-plate (~800rpm)
- An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle.
- 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).

9. Preparation of reagents

- ✧ Bring all reagents of the kit to room temperature before use.
- ✧ Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.
- ✧ Do not use the reagent after expiration date.

● Reagents ready for use after return to room temperature

[Antibody-coated plate]

Storage and stability

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.

[Buffer solution]

[Chromogenic substrate reagent]

Storage and stability

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

[Reaction stopper (1 M H_2SO_4)]

Storage and stability

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

● Concentrated reagents

[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 dionized water.

Storage and stability

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

Dispose any unused diluted buffer.

[IgE Standard solution (100 ng/ml)]

Make a serial dilution of master standard (100 ng/ml) solution to prepare each standard solution.

Volume of standard solution	Buffer solution	Concentration (ng/ml)
Original solution	0 µl	100
Original solution 150 µl	50 µl	75
Original solution 100 µl	100 µl	50
Original solution 50 µl	150 µl	25
Original solution 20 µl	180 µl	10
Original solution 5 µl	495 µl	1.0
0 (Blank)	200 µl	0

Storage and stability

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

The rest of original standard: if stored tightly closed at 2-8 °C, it is stable until expiration date.

[Biotin-conjugated anti-IgE antibody]

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

Storage and stability

Unused working solution (already diluted) should be disposed.

The rest of the undiluted solution: if stored tightly closed at 2-8 °C, it is stable until expiration date.

[HRP-conjugated avidin]

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

Storage and stability

Unused working solution (already diluted) should be disposed.

The rest of the undiluted solution: if stored tightly closed at 2-8 °C, it is stable until expiration date.

10. Preparation of samples

This kit is intended to measure IgE in rat serum or plasma.

Samples should be immediately assayed or stored below –35 °C until assay. Dilution of a samples should be made in a test tube using buffer solution prior to adding them to wells. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter. Hemolytic and hyperlipemic serum samples are not suitable. If presence of interfering substance is suspected, examine by dilution test at more than 2 points.

Storage and stability

Use samples soon after collected. Collected samples can be stable within 1 week after collection 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. Defrosted samples should be mixed thoroughly for best results. Avoid repeated freeze-thaw cycles.

●Testing for compatibility of your samples with Shibayagi's kit using a positive sample.

Due to various factors of your sampling conditions (anesthesia, preservatives, anticoagulants, raised sample pH caused by loss of CO_2 during standing and storage, preservative used, evaporation and condensation during storage in a freezer, etc), sometimes the kit does not work well with your samples. If the standard curve is in a good shape, while your samples give low absorbance, please check the compatibility of your samples (serum or plasma) by a simple recovery test as follows.

Place 90 µl of your sample (e.g. a sample from control group in your experiment) in a small test tube, then add 10 µl of the highest standard solution (10ng/ml). Assay this mixture together with the original sample, and compare the assay values. The assay value of the mixture will be around [0.9 x original sample + 0.1 x highest standard concentration]. If the assay value is increased as expected, the assay system is working well with your sample.

Especially when you use Shibayagi's kit for the first time, we recommend you to run this simple recovery test.

●Quality control samples

We recommend preparing quality control samples of your own laboratory by storing many aliquots of serum, plasma or culture medium with known amount of the analyte to be measured after initial testing. Keep them in small and tightly capped sample tubes below －35 °C. If the sample tube is too big, water will be lost during storage. If possible, prepare high and low controls.

Measure these control samples along with your samples in every run to confirm the reproducibility and successful performance of the assay system.

11. Assay procedure

Remove the cover sheet of the antibody-coated plate after bringing up to room temperature.

(1) Wash the antibody-coated plate (A) by filling the wells with washing buffer and discard 3 times, then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(2) Pipette 50 µl of properly diluted samples to the designated sample wells.
(3) Pipette 50µl of standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker (800rpm for 10 seconds x 3 times).
(5) Pipette 50µl of Biotin-conjugated anti-IgE antibody to all wells, and shake as step (4).

(6) Put a plate cover on the plate and incubate for 2 hours at room temperature (20-25°C).
(7) Discard the reaction mixture. Rinse wells by filling the wells with washing buffer and discard 3 times, then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(8) Pipette 100µl of HRP-conjugated avidin to all wells, and shake as step (4).
(9) Put a plate cover on the plate and incubate the plate for 1 hour at room temperature.
(10) Discard the reaction mixture. Rinse wells by filling the wells with washing buffer and discard 3 times, then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(11) Pipette 100µl of Chromogenic substrate reagent to wells, and shake as step (4).
(12) Put a plate cover on the plate and incubate the plate for 20 minutes at room temperature.
(13) Add 100 µl of the reaction stopper to all wells and shake as step (4).
(14) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

Note: For manual washing procedure see "Kit operation (Power point)" or "Shibayagi's Manual Operation" at http://www.shibayagi.co.jp/

*600-650nm can be used as reference wavelength. For washing of the plate, an automatic plate washer is preferable, however, a washing bottle with a jet nozzle can be used. A syringe type repeating dispenser like Eppendorf's multipet plus set at 300 µl is also useful.

Standard of plate-washer pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Be careful not to make the well-plate dry.

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	**100 ng/ml**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	**75 ng/ml**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	**50 ng/ml**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	**25 ng/ml**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	**10 ng/ml**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	**1.0 ng/ml**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	**0**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	**Positive Control**	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

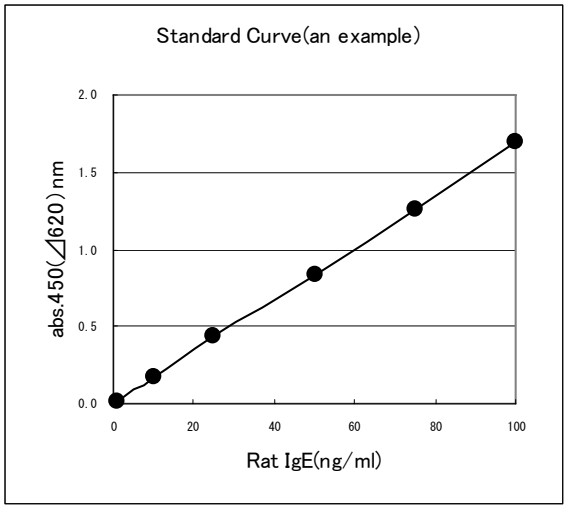
12. Calculations

(1) Prepare a standard curve by plotting standard concentration on X-axis and absorbance on Y-axis.
(Refer to our web site for more detailed explanation about standard curve. Shibayagi is offering a convenient Excel template. http://www.shibayagi.co.jp/en/tech_003.html)

(2) Using the standard curve, read the IgE 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, 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.

Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.



Rat IgE assay standard curve (an example)

Absorbance may change due to assay environment.

13. Performance characteristics

- Assay range

The assay range of the kit is 1 ng/ml ~ 100 ng/ml.

- Specificity

The antibodies used in this kit are specific to rat IgE. Cross-reactivity of the kit is shown below.

Substances	Cross-reactivity	Concentration
Rat IgE	100%	100 ng/ml
Rat IgG	< 0.01%	1 mg/ml
Rat IgA	< 0.01%	1 mg/ml
Rat IgM	< 0.01%	1 mg/ml
Mouse IgE	< 0.1%	100 ng/ml
Human IgE	< 0.1%	100 ng/ml
BSA	< 0.01%	10 mg/ml

- Precision of assay

Within assay variation (3 samples, 5 replicates assay,)
Mean CV is less than 5%.

- Reproducibility

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

- Recovery test

Standard IgE was added in 4 concentrations to 2 serum samples and were assayed.
The recoveries were 97.9 ~107%

- Dilution test

Two serum samples were serially diluted and assayed in 3 replicates.
The dilution curves showed linearity with $R^2 = 0.998$ and 0.999.

14. Reference assay data

Rat IgE assay data

Mean assay value: 45.6 ng/ml, SD: 6.2 ng/ml

Rat strains: CD (SD), male, 5 weeks-old
Number of animals: 7 Samples: serum

These data should be considered as guidance only. Each laboratory should establish its own normal and pathological reference ranges for IgE 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-conjugated anti-IgE antibody, HRP-conjugated avidin, or Chromogenic substrate reagent might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-conjugated anti-IgE antibody or HRP-conjugated avidin.
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 chromogenic substrate reagent soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

- Blank OD was higher that that of the lowest standard concentration (1.0 ng/ml).

Possible explanations:

Improper or inadequate washing. (Change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP-conjugated 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).

16. References

Please, refer to "User's Publication" on our website.

Summary of assay procedure

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

☐Bring the well-plate and all reagents back to room temperature.

☐ Concentrated washing buffer must be diluted to 10 times by purified water that returned to room temperature.

☐IgE Standard solution dilution example:

Concentration (ng/ml)	100	75	50	25	10	1.0	0
IgE Std. solution (µl)→	Ori.Sol.	150	100	50	20	5	0
Buffer solution (µl)		50	100	150	180	495	200

☐Prepare the positive sample.

☐Biotin-conjugated anti-IgE antibody: Dilute it to 100x using the buffer solution returned to room tepm.

☐	**Antibody-coated plate**
☐	**↓Washing 3 times***
☐	**Diluted samples, or Standards 50 µl**
☐	**↓Shaking****
☐	**Bioton-conjugated anti-IgE antibody solution 50 µl**
☐	**↓ Shaking**, Incubation for 2 hours at room temp.** (Standing***)
☐	Dilute HRP-conjugated avidin to 100x with buffer returned to room temp. (This should be prepared during incubation.)
☐	**↓Washing 3 times***
☐	**HRP conjugated avidin 100 µl**
☐	**↓ Shaking**, Incubation for 1 hour at room temp.** (Standing***)
☐	**↓Washing 3 times***
☐	**Chromogenic substrate reagent (TMB) 100 µl**
☐	**↓ Shaking**, Incubation for 20 minutes at room temp.** (Standing***)
☐	**Reaction stopper (1M H_2SO_4) 100 µl**
☐	**↓Shaking****
☐	**Measurement of absorbance (450nm, Ref 620nm) Use the value (abs.450nm－abs.620nm)**

☐ : Use as a check box **Room temp: 20~25℃**

* Guideline of washing volume: 300µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. If the back ground is high, change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP conjugated streptavidin. Be careful not to make the well-plate dry.

** Guideline of shaking: 800rpm for 10 seconds x 3 times. 600-650 nm can be used as reference wavelength.
***Put a plate cover on the plate while the reaction after shaking.

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 2-8C (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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