For Research Use Only December 10, 2012 Ver. 5

[Mouse anti-OVA-IgE ELISA Kit]

(Code No.:AKRIE-030)

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

Mouse anti-OVA-IgE ELISA Kit is an ELISA system for quantitative measurement of mouse anti-OVA-IgE antibody titer. This is intended for research use only.

2. Storage and expiration

When the intact 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

IgE (immunoglobulin E) is the 5th immunoglobulin found and is composed of 2 heavy chains which contain 5 domains (VH, CHε 1-4) and 2 light chains. It is a glycoprotein with molecular weight of 190kDa, and in electrophoresis it moves to γ1 region. Metabolic half life of IgE is about 3 days in man, and normal serum level in human is about 300ng/ml, however, it is markedly increased in parasite infection and hay fever. Allergy-related IgE is called reagin. The Fc region of reagin increased after sensitization with allergens will bind Fcε R1 receptor of basophilic granulocytes and mast cells in the skin, respiratory, and digestive organs, and sensitizes those cells. Those IgE-sensitized cells will be degranulated when the second allergens bind the surface IgE, and release histamine, serotonine, protease, heparin, chemotactic factors, prostaglandins, leucotriens, etc., causing type I allergy reactions.

Shibayagi's OVA-IgE ELISA KIT is a useful tool for studying mouse immune system by measuring specific anti-OVA IgE after immunization of mice with OVA (ovalbumin).

4. Assay principle

In Shibayagi's Mouse anti-OVA-IgE ELISA Kit, biotin-conjugated anti-mouse IgE antibody, standards or samples are incubated in OVA-coated wells to capture OVA-IgE. After 1 hour incubation and washing, HRP (horse radish peroxidase)-labeled avidin is added, and incubated for 30 minutes together with captured anti-mouse OVA-IgE antibody. After washing, HRP-complex remaining in wells is 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 nearly proportional to anti-mouse OVA- IgE antibody titer. The standard curve is prepared by plotting absorbance against standard OVA-IgE concentrations. The 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) OVA-coated 96 well-plate (Dried-plate)	Ready for use.	96 wells/1 plate
(B) Mouse anti-OVA- IgE standard (1200 U/ml) (derived from mouse)	Concentrated. Use after dilution.	100 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-conjugated anti-mouse IgE antibody	Concentrated. Use after dilution.	200 µl/1 vial
(E) HRP-avidin conjugate	Concentrated. Use after dilution.	200 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	−	3 sheets
Instruction Manual	−	1 copy

7. Equipments 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 10-100µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 µl and 100µl.
☐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) Mouse anti-OVA-IgE standard (1200 U/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(U/ml)
Original solution : 10 µl	90 µl	120
120 U/ml solution : 50 µl	50 µl	60
60 U/ml solution : 50 µl	50 µl	30
30 U/ml solution : 50 µl	50 µl	15
15 U/ml solution : 50 µl	50 µl	7.5
7.5 U/ml solution : 50 µl	50 µl	3.75
3.75 U/ml solution : 50 µl	50 µl	1.88
0 (Blank)	50 µl	0

*In this kit, 1 U/ml is prescribed to antigen-binding constant (Ka) $2.0 \times 10^{8} M^{-1}$ antibody 1.3 ng/ml.

[(D) Biotin-conjugated anti-mouse IgE antibody]

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) OVA-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) Mouse anti-OVA-IgE standard (1200 U/ml)]

Standard solutions prepared above should be used as soon as possible, and should not be stored. Unused working solution (already diluted) should be disposed.

[(C) Buffer solution] & [(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-conjugated anti-mouse IgE 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

- 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 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].
- The standard of this kit is anti-OVA-IgE monoclonal antibody. Therefore, it is possible to compare the assay results even if this kit is used in a different laboratory. Principally it is not possible to compare the assay results with other assay kits' results because the standards don't always have the affinity to the same OVA.

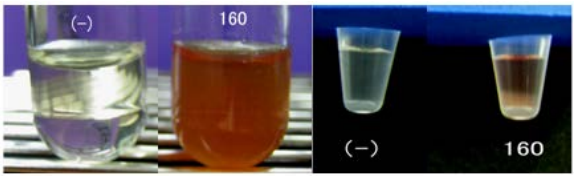
10. Preparation of samples

This kit is intended to measure anti-mouse OVA-IgE antibody titer in mouse serum or plasma.

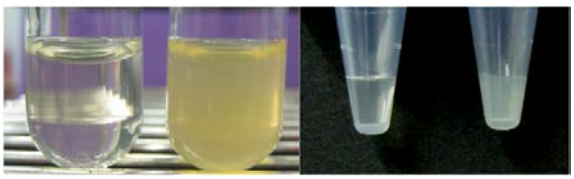
Samples should be immediately assayed or stored below –35 °C for several days. Defrosted samples should be mixed thoroughly for best results.

Hemolytic and hyperlipemic 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 160mg/dL with this kit.



Normal | Hemolysis 160mg/dL | normal | Hemolysis 160mg/dL



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

If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

Make sure to dilute samples more than 10x. Recommended is 10-50x depending on the antibody titer. Dilution should be carried out with the buffer solution of the kit using small test tubes before assay.

Example of dilution: Rate	10x		20x		50x
Sample (µl)	5	⌐	25*	⌐	20*
Buffer (µl)	45	⌊	25	⌊	30

*One rank lower diluted sample

Storage and stability

Sample is stable at 2-8°C within a week. 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

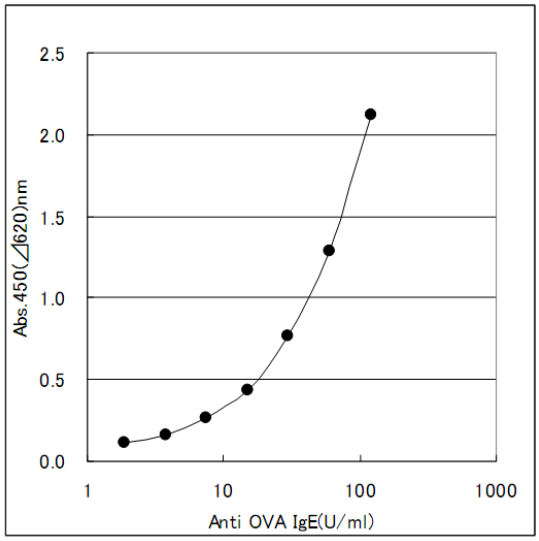
Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

(1) Wash the antibody 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 biotin conjugated anti-mouse IgE antibody (D) to the designated wells. Shake the plate gently on a plate shaker (*③).
(3) Pipette 10 µl of diluted samples to the wells designated for samples.
(4) Pipette 10 µl of standards to the wells designated for standards.
(5) Shake the plate on a plate shaker (*③).
(6) Stick a plate seal (*④) on the plate and incubate for 1 hour at 20-25°C.
(7) Discard the reaction mixture and rinse wells as step (1).
(8) Pipette 100 µl of HRP-conjugated avidin solution (E) to all wells, and shake as step (5).
(9) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(10) Discard the reaction mixture and rinse wells as step (1).
(11) Pipette 100 µl of chromogenic substrate solution (F) to wells, and shake as step (5).
(12) Stick a plate seal (*④) on the plate and incubate the plate for 20 minutes at 20-25°C.
(13) Add 100 µl of the reaction stopper (H) to all wells and shake as step (5).
(14) 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 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 anti-OVA-IgE concentration (U/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 anti-OVA-IgE 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. Clinical findings in mouse should be judged collectively considering clinical manifestation or other test results.



Mouse ant-OVA-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 1.88 ~ 120 U/ml.

- Specificity

The biotin conjugated anti-mouse IgE antibody of this kit is specific to mouse IgE.

- Precision of assay

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

- Reproducibility

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

- Recovery test

Anti-mouse OVA-IgE was added in 3 concentrations to 2 serum samples and was assayed.
The recoveries were 95.8 ~ 106%

● Dilution test

2 serum samples were serially diluted by 3 steps.
The dilution curves showed excellent linearity. (R^2= 0.9987 ~ 0.9999)

14. Reference assay data

Mouse OVA-IgE antibody titer's mean assay value: 139 U/ml, SD: 22.5 U/ml
Strain: BALB/c, 3 males, 8 week-old
OVA administration: Equal volumes of alum (20mg/ml) and OVA (50μg/ml) were mixed, and 0.2ml/head of the mixture was intraperitoneally injected twice with 1 week interval, then blood sampling was made 3 weeks later. OVA was first solubilized with 0.1M carbonate buffer pH 8.5 at a concentration of 1mg/ml, then diluted with saline to make 50μg/ml.
These data should be considered as guidance only. Each laboratory should establish its own normal and pathological reference ranges for OVA-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 antibody, HRP-conjugated avidin, or TMB might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-conjugated 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.

● Blank OD was higher than that of the lowest standard concentration (1.88 U/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 anti-OVA-IgE antibody.)

● 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 96 well-plate is empty when I opened the box.

A-2: As this kit is dried type, not preservation stabilizer is added.

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 ☐ : 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.

☐Standard solution dilution example:

Concentration (U/ml)		120	60	30	15	7.5	3.75	1.88	0
Std. solution (µl)	Orig.sol. 10		50*	50*	50*	50*	50*	50*	0
Buffer solution (µl)		90	50	50	50	50	50	50	50

*One rank higher standard.

☐Make the positive control.

☐Dilute biotin-conjugated anti-mouse IgE antibody to 100x with buffer returned to 20-25°C.

			Precautions & related info
☐	OVA-coated 96 well-plate (Dried-plate)		
☐	↓ Washing 3 times(*②)		*⑥
☐	Biotin-conjugated anti-mouse IgE antibody	50 µl	***⑦ [Handling of pipetting]**
☐	↓ Shaking(*③)		
☐	Diluted Samples / Standards	10 µl	***⑦ [Handling of pipetting]**
☐	↓ Shaking(*③), Incubation for 1 hour at room temp. (Standing(*④))		***⑧ [Assay circumstance]**
☐	Dilute HRP-conjugated avidin (E) to 100x with buffer (C) returned to 20-25°C.		
☐	↓ Washing 3 times(*②)		*⑥
☐	HRP-conjugated avidin	100 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)	100 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 20 mins 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. 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	**120 U/ml**	Pos. Control	Sample 8	Sample 16	Sample 24	Sample 32
B	**60 U/ml**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	**30 U/ml**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	**15 U/ml**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	**7.5 U/ml**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	**3.75 U/ml**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	**1.88 U/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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