For Research Use Only March 15, 2013 Ver. 1

[Mouse Insulin ELISA Kit (RTU)]

(Code No.:AKRIN-011RU)

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 Insulin ELISA Kit (RTU) is a sandwich ELISA system for quantitative measurement of mouse insulin. This is intended for research use only. In this kit, ready for use standard solutions are provided. Serial dilution by your own hands is no longer necessary. Other reagents and assay buffer can be also used without dilution. This will save your labor and increase assay performance as well.

2. Storage and expiration

When the complete kit is stored at 2-8°C (Do not freeze), the kit is stable until the expiration date shown on the label on the container. Opened reagents should be used as soon as possible to avoid less than optimal assay performance caused by storage environment.

3. Introduction

Insulin is a peptide hormone secreted from B cells of islet of Langerhans in the pancreas with a molecular weight of about 5,800 and pI 5.4. It is consisted of 2 chains, A and B. It has 3 disulfide bonds formed between A6 and A11, A7 and B7, and A20 and B19. Insulin exists as a dimer molecule in acidic to neutral solution without Zn ion, and as a hexamer including two Zn ions in neutral solution if Zn ions are present. Main targets of insulin are liver, muscle, and adipose tissue. Insulin actions in these targets are as follows. In the liver, it promotes glycogenesis, protein synthesis, fatty acid synthesis, carbohydrate utilization, and inhibition of gluconeogenesis.

In the muscle, it promotes membrane permeability for carbohydrates, amino acids and K ion, glycogenesis, protein synthesis, while inhibits protein degradation. In the adipose tissue, it promotes membrane permeability for glucose and fatty acid synthesis. A precursor of insulin, called proinsulin with a single polypeptide chain, is first synthesized in the cell, then sulfide bonds are formed, and finally by enzymatic cutting at two sites, active insulin and c-peptide (connecting peptide) are formed. Potency of an insulin preparation was originally determined by bioassay. However, whole body bioassay inevitably shows poor precision owing to individual variation.

WHO issued 1st International Standard for human insulin in 1986 which has the potency of 26 IU/mg (0.038 mg/IU). In the same year, 1st International Standard of bovine insulin, the potency of which is 25.7 IU/mg, and Porcine insulin 1st International Standard, 26 IU/mg, were provided. Before these standards, in 1974, 1st International Reference Preparation of human insulin for immunoassay was provided as 3 IU/ampoule. Based on the above data, if the biological activity of insulin per molecule is the same among various animal species, potencies of animal insulin might be calculated from their molecular weights. But, so far, we do not have experimental proof about this. As the molecular weights of insulin of various animals are nearly the same, and the differences are within 1%, there may be no critical fault if we think that the general potency of insulin is 26 IU/mg. Rat and mouse have two molecular species of insulin, type 1 and type 2. Amino acid sequences of these molecular species are same between rat and mouse. But as their ratios are different between these two animal species, it is recommended to use standard preparation derived from each animal.

4. Assay principle

In Shibayagi's Mouse Insulin ELISA Kit (RTU), biotin conjugated anti-insulin, and standard or sample are incubated in monoclonal anti-insulin-coated wells to capture insulin bound with biotin conjugated anti-insulin. After 2 hours' incubation and washing, HRP (horse radish peroxidase) conjugated streptavidin is added, and incubated for 30 minutes. After washing, HRP conjugated streptavidin remaining in wells are reacted with a substrate chromogen 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 proportional to insulin concentration. The standard curve is prepared by plotting absorbance against standard insulin concentrations. Insulin 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. In manual operation, proficiency in pipetting technique is recommended.
- Use clean laboratory glassware.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- 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 1M 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.
- 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.
- 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 materials must not be pipetted by mouth.
- 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)Anti-Insulin-coated plate	Use after washing	96 wells/1 plate
(B)Standard Mouse Insulin solutions (derived from mouse) (6 concentrations, pg/ml: color) ①12,000:● ②4,800:● ③2,000:● ④800:● ⑤300:● ⑥100:○	Ready for use.	100µl/1 vial/conc. (6 conc., 6 vials)
(C)Buffer solution	Ready for use.	60 ml/1 bottle
(D)Biotin conjugated anti-insulin	Ready for use.	12 ml/1 bottle
(E)HRP conjugated streptavidin	Ready for use.	12 ml/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	—	3 sheets
Instruction Manual	—	1 copy

7. Equipments or supplies required but not supplied　☐Use as a check box

☐Purified water (distilled water)
☐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 100 µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 100 µl.
☐Paper towel to remove washing buffer remaining in wells.
☐A vortex-type mixer.
☐A shaker for 96 well-plate (600-1,200rpm)
☐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) for about 2 hours before use.
◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents. The reagents indicated as ready for use in section 6 can be used without preparation after getting back to room temperature.

【Concentrated reagents】

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

Dilute 1 volume of the washing buffer concentrate (10x) to 10 volumes with deionized water to prepare working solution. Example: 100 ml of washing buffer concentrate (10x) and 900ml of dionized water.

【Storage and stability】

[(A)Anti-Insulin-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 Mouse Insulin solutions]

When you separate the kit for plural assays, take out the standard solution vials from refrigerator just before assay. To avoid contamination, exchange tips at each delivery. Remaining standards should be tightly capped and stored at 2-8 °C before returning to 20-25 °C. They will be stable until expiration date.

[(C)Buffer solution], [(D)Biotin conjugated anti-insulin], [(E)HRP conjugated streptavidin] & [(F)Substrate chromogen reagent(TMB)]

When separating the kit for plural assays, transfer a little more volume than necessary to other vessels and the remaining solutions should be tightly capped and stored at 2-8 °C before returning to20-25 °C. They will maintain stability until expiration date.

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

The remaining solution should be tightly capped and stored at 2-8 ° C. It will maintain stability until expiration date.

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

The remaining solution should be tightly capped and stored at 2-8 °C. It will maintain stability until expiration date. Dispose any unused diluted buffer.

9. Technical tips

●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 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.
●Time the reaction from the pipetting of the reagent to the first well.
●The substrate chromogen 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.

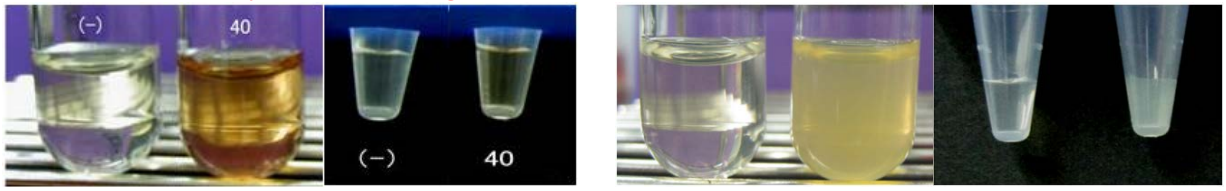
●When ELISA has to be done under the airstream velocity over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

10. Preparation of samples

This kit is intended to measure insulin in mouse serum, plasma, culture medium and tissue/cell extracts. The necessary sample volume for the standard procedure is 10 µl. 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 serum 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 40mg/dL with this kit.



Normal | Hemolysis 40mg/dL | normal | Hemolysis 40mg/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. Dilution of a sample 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.

Storage and stability

Insulin in samples will be inactivated if stored at 2-8ºC. If it is necessary to store sample in refrigerator (2-8ºC), add aprotinin at final concentration of 100-500KIU/ml. (KIU: kallikrein inhibitor unit).

If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35ºC. 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, plasma, or culture medium) 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 anti-Insulin-coated plate after bringing up to room temperature.

(1) Wash the anti-Insulin-coated plate (A) by filling the wells with 300 µl of washing buffer and discard 4 times(*②), then strike the plate upside-down onto several sheets of paper towel to remove residual buffer in the wells.
(2) Pipette 100µl of biotin conjugated anti-insulin to all wells. Shake the plate gently on a plate shaker(*③).
(3) Pipette 10µl of sample to the designated sample wells.
(4) Pipette 10µl of standard solutions to the wells designated for standards..
(5) Shake the plate gently on a plate shaker(*③) .
(6) Stick a plate seal (*④) 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 300 µl of washing buffer and discard 4 times(*②), then strike the plate upside-down onto folded several sheets of paper towel to remove residual buffer in the wells.
(8) Pipette 100µl of HRP conjugated streptavidin 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 room temperature.
(10) Discard the reaction mixture, and then wash the plate as step (1) .
(11) Pipette 100µl of substrate chromogen reagent to wells, and shake as step (5).
(12) Stick a plate seal (*④) 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 (5) .
(14) Measure the absorbance of each well at 450 nm (reference wavelength, 620nm) using a plate reader within 30 minutes. 600-650nm can be used as reference wavelength.

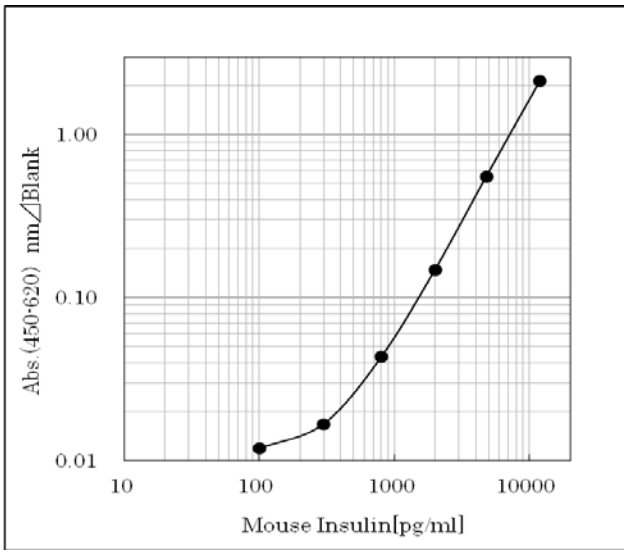
*Refer to the page 7-8 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve for each assay. Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance(Y-axis) against insulin concentration (ng/ml) on X-axis.

*Absorbance at 450nm minus absorbance at 620nm.

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



Mouse insulin assay standard curve (an example)
Absorbance may change due to assay environment.

(3) If the reaction temperature is high, the absorbance may be high on the whole. Don't use any standard curve area with less reliable absorbance, and repeat the assay adjusting the temperature at 20-25 °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. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.

**Reference for unit conversion; µIU/ml = 26 IU/mg. See also section 3.

Concentration (pg/ml)	Concentration (µIU/ml**)
12,000	312.0
4,800	124.8
2,000	52.0
800	20.8
300	7.8
100	2.6

13. Performance characteristics

●Assay range; The assay range of the kit is 100 ~ 12,000 pg/ml.

●Specificity; The antibodies used in this kit are specific to insulin.

Cross-reactivity of the kit is shown below. Cross-reactivity at Conc. 12,000 pg/ml.

Substances	Cross-reactivity	Substances	Cross-reactivity
Mouse C-peptide	−	Rat insulin	+
Mouse proinsulin	+	Human insulin	+

●Precision of assay

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

●Reproducibility

Between assay variation (three samples, four days, assayed in triplicate); Mean CV is less than 5%

●Recovery test

Standard insulin was added in four concentrations to two serum samples and were assayed. The recoveries were 96 ~106%

●Dilution test

Two serum samples were serially diluted by four steps. The dilution curves showed linearity with $R^2 = 0.998 \sim 0.999$.

14. Reference assay data

Mouse insulin assay data: 798 ~ 2,425 pg/ml

Mouse strains: C57BL/6, BALB/c, male, 8 weeks old, fed *ad libitum*

Number of animals: 12/strain　Samples: sera

These data should be considered as guidance only. Each laboratory should establish its own normal and pathological reference ranges for insulin 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 insulin, HRP conjugated streptavidin, or Substrate chromogen reagent might not be added.
3) Wrong reagents related to coloration might have been added.
4) Contamination of enzyme inhibitor(s).
5) Influence of the temperature under which the kits had been stored (if stored frozen).
6) Excessive hard washing of the well plate.
7) Addition of substrate chromogen reagent 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 (100 pg/ml).

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 conjugated streptavidin.)

● High coefficient of variation (CV)

Possible explanation:

1) Improper or inadequate washing.
2) Improper mixing of standard or samples (Sufficiently mix frozen samples).
3) Pipetting operation was not constant.

● Q-1: Can I divide the plate to use it for the other assay?

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 add 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 ☐ : 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 back to room temperature at 20~25 °C for 2 hours.

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

☐Prepare the positive sample.

			Precautions & related info
☐	Anti-Insulin-coated plate		
☐	↓Washing 4 times(*②)		*⑥
☐	Biotin conjugated anti-insulin	100 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③)		
☐	Samples/Standards	10 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 2 hours at 20-25oC. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓Washing 4 times(*②)		*⑥
☐	HRP conjugated streptavidin	100 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 30 minutes at 20-25oC. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓Washing 4 times(*②)		*⑥
☐	Substrate chromogen reagent (TMB)	100 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓Shaking(*③), Incubation for 20 minutes at 20-25oC. (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 swirl the plate on your palm for 10 sec and shake off the buffer. After successive four-times-washing, strike the plate upside-down onto several layers of paper towels to remove residual buffer remaining in the wells. Immediately apply next reagent so as not to let the wells go dry.
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.) Be careful with the contamination between wells at the only initial washing after the first reaction. 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	12,000 pg/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	4,800 pg/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	2,000 pg/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	800 pg/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	300 pg/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	100 pg/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	0	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	Pos.Control.	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

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] 12 months from production (Expiration date is indicated on the container.)

This kit is manufactured by **Shibayagi Co., Ltd.**
1062-1 Ishihara, Shibukawa, Gunma, Japan 377-0007
TEL.+81-279-25-0279, FAX.+81-279-23-0313
URL:http://www.shibayagi.co.jp/ E-mail:syc-info@shibayagi.co.jp