FUJIFILM LBIS

For Research Use Only

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[LBIS Porcine Insulin ELISA Kit]

Cat # 634-01469 (Manufacture # AKRIN-013T)
Please, read this instruction carefully before use.

This kit is manufactured by FUJIFILM Wako Shibayagi Corporation. 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.

1. Intended use

LBIS Porcine Insulin ELISA Kit is a sandwich ELISA system for quantitative measurement of porcine insulin. This 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 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

Insulin is a peptide hormone secreted from B cells of islet of Langerhans in the pancreas with a molecular weight of about 5800 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 animals.

4. Assay principle

In Shibayagi's LBIS Porcine Insulin ELISA Kit, biotinylated anti-insulin antibody, and standard or sample are incubated in monoclonal anti-insulin coated wells to capture insulin bound with biotinylated anti-insulin antibody. 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 chromogen (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 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 stop solution and chromogen (TMB) containing hydrogen peroxide and tetramethylbenzidine. 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 stop solution because it is 1M sulfuric acid. The stop solution 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 egional/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 Porcine Insulin solution (240 ng/mL) (derived from porcine pancreas extract)	Concentrated. Use after dilution	25 µL/1 vial
(C) Buffer solution	Ready for use.	60 mL/1 bottle
(D) Biotinylated anti-insulin antibody	Concentrated. Use after dilution.	10 µL/1 vial
(E) HRP-conjugated streptavidin	Concentrated. Use after dilution.	20 µL/1 vial
(F) Chromogen (TMB)	Ready for use.	12 mL/1 bottle
(H) Stop solution (1 M H_2SO_4) Be careful!	Ready for use.	12 mL/1 bottle
(I) Wash stock solution (10×)	Concentrated. Use after dilution.	100 mL/1 bottle
Plate seal	—	3 sheets
Instruction Manual	—	1 copy

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

☐Deionized water (or Distilled water) ☐Test tubes for preparation of standard solution series.
☐Glassware for dilution of Wash stock solution (10×)(a graduated cylinder, a bottle).
☐Pipettes (disposable tip type). One should be able to deliver 10 µL precisely, and another for 100-200 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 (600-1200 rpm). ☐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 (450 nm ±10 nm, 620 nm: 600 nm -650) ☐Software for data analysis.

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) Standard Porcine Insulin solution (240 ng/mL)]
Make a serial dilution of master standard (240 ng/mL) solution to prepare each standard solution.

Volume of standard solution	Buffer solution	Concentration (ng/mL)	Concentration (μIU/mL)
Original solution: 10 μL	190 μL	12	312
12 ng/mL solution: 100 μL	100 μL	6.0	156
6.0 ng/mL solution: 100 μL	100 μL	3.0	78
3.0 ng/mL solution: 100 μL	100 μL	1.5	39
1.5 ng/mL solution: 100 μL	100 μL	0.75	19.5
0.75 ng/mL solution: 100 μL	100 μL	0.375	9.75
0.375 ng/mL solution: 100 μL	100 μL	0.188	4.89
0 (Blank)	100 μL	0	0

[(D) Biotinylated anti-insulin antibody]
Prepare working solution by dilution of (D) with the buffer solution (C) to 1:4000.
10 mL of the diluted solution is enough for 96 wells.
[(E) HRP-conjugated streptavidin]
Prepare working solution by dilution of (E) with the buffer solution (C) to 1:2000.
10 mL of the diluted solution is enough for 96 wells.
[(I) Wash stock solution (10×)]
Dilute 1 volume of the concentrated Wash stock solution (10×) to 10 volume with deionized water (or distilled water) to prepare working solution. Example: 100 mL of concentrated washing buffer (10×) and 900 mL of deionized water (or distilled 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 Porcine Insulin solution (240 ng/mL)]
Standard solutions prepared above should be used as soon as possible, and should not be stored. *Unit reduction for μIU/ml is 26 IU/mL. (Refer to 3. Introduction.)
[(C)Buffer solution]& [(F)Chromogen (TMB)]
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)Biotinylated anti-insulin antibody] &[(E)HRP-conjugated streptavidin]
Unused working solution (already diluted) should be disposed.
[(H) Stop solution (1 M H_2SO_4)]
Close the stopper tightly and store at 2-8 °C. It maintains stability until expiration date.
[(I) Wash stock solution (10×)]
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

- 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.
- 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.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogen (TMB) should be almost clear pale blue before use. It turns blue during reaction, and gives yellowish color after addition of stop solution. 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 8 wells × 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 %,

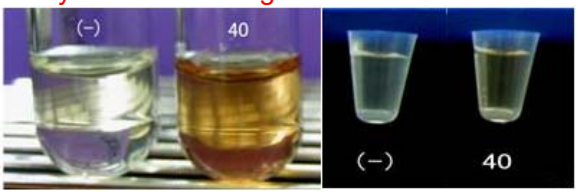
seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

10. Preparation of samples

This kit is intended to measure insulin in porcine serum, plasma (preferably obtained with heparin), 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 40 mg/dL with this kit.



Normal | Hemolysis 40 mg/dL | normal | Hemolysis 40 mg/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 2-8 ºC. If it is necessary to store sample in refrigerator (2-8 ºC), add aprotinin at final concentration of 100-500 KIU/mL. (KIU: kallikrein inhibitor unit).

If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35 °C. Avoid repeated freeze-thaw cycles.

11. Assay procedure

Remove the cover sheet of the anti-Insulin-coated plate after bringing back 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 layers of paper towels to remove residual buffer remaining in the wells.
(2) Pipette 100 µL of biotinylated anti-insulin antibody to all wells. Shake the plate gently on a plate shaker(*③).
(3) Pipette 10 µL of sample to the wells designated for samples.
(4) Pipette 10 µL of standard solution 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 20-25 °C.
(7) Discard the reaction mixture, and then wash the plate as step (1)
(8) Pipette 100 µL 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 20-25 °C .
(10) Discard the reaction mixture, and then wash the plate as step (1).
(11) Pipette 100 µL of chromogen (TMB) to wells, and shake as step (5).
(12) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25 °C .
(13) Add 100 µL of the stop solution to all wells and shake as step (5).
(14) Measure the absorbance of each well at 450 nm (reference wavelength, 620 nm*) by a plate reader within 30 minutes.

*Refer to the page 7 for notes of *②,*③and (*④).

12. Calculations

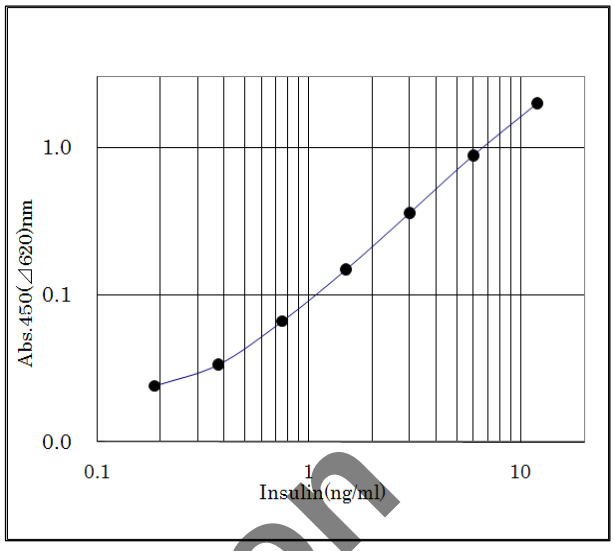
(1) Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against insulin concentration (ng/mL) on X-axis.

*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.

* We recommend the use of 3rd order regression curve for log-log plot, or 4 or 5 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.



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

13. Performance characteristics

●Assay range

The assay range of the kit is 0.188 ~ 12 ng/mL.

●Specificity

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

Substances	Cross-reactivity	Substances	Cross-reactivity
Porcine Insulin	100 %	Rat Insulin	83 %
Porcine C-peptide	—	Human Insulin	155 %
Mouse insulin	85 %		

*Cross-reactivity at Conc. 12 ng/mL.

For details, refer to: http://www.shibayagi.co.jp/en/pdf/InsulinSpecificity.pdf

●Precision of assay

Within assay variation (3 samples, 5 replicates assay) Mean CV was within 10 %.

●Reproducibility

Between assay variation (3 samples, 3 days, assayed in duplicates) Mean CV was within 10 %

●Recovery test

Standard insulin was added in 4 concentrations to 2 serum samples and were assayed.
The recoveries were 91.7 ~ 102 %

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 biotinylated anti-insulin antibody, HRP-conjugated streptavidin, or chromogen (TMB) might not be added.

3)Wrong reagents related to coloration might have been added. Wrong dilution of biotinylated anti- insulin antibody or HRP-conjugated streptavidin.

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 chromogen (TMB) soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

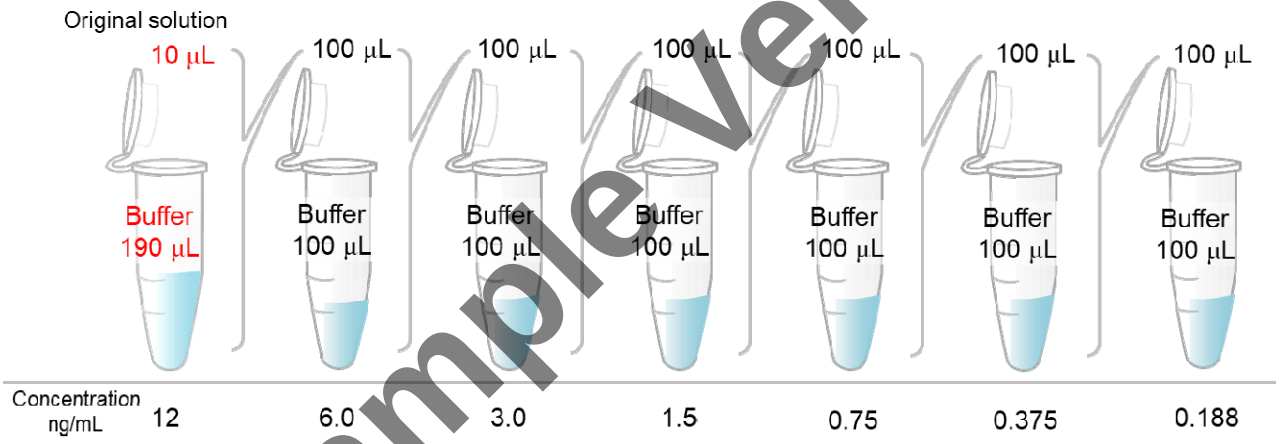
●The OD of blank is higher than that of the lowest standard concentration (0.188 ng/mL)

Possible explanations:

Improper or inadequate washing. (Change washing repetition from 4 times to 5-8 times 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.
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
*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.
☐Wash stock solution (10×) concentrate must be diluted to 10 times by deionized water (or distilled water that returned to 20-25 °C.
☐Standard Porcine Insulin solution dilution example:



☐Biotinylated anti-insulin antibody(D) : Dilute to 4000 times by using buffer solution(C) and use.

☐	Anti-Insulin coated plate		
☐	↓Washing 4 times(*②)		*⑥
☐	Biotinylated anti-insulin antibody	100 µL	*⑦
☐	↓Shaking(*③)		
☐	Samples/Standards	10 µL	*⑦
☐	↓Shaking(*③), Incubation for 2 hours at 20-25 °C. (Standing(*④))		*⑧
☐	HRP-conjugated streptavidin(E) Dilute to 2000 times by using buffer solution(C) and use. (Dilute reagents during the first reaction. 2-step dilution is recommended.)		
☐	↓Washing 4 times(*②)		*⑥
☐	HRP-conjugated streptavidin	100 µL	*⑦
☐	↓Shaking(*③), Incubation for 30 minutes at 20-25 °C. (Standing(*④))		*⑧
☐	↓Washing 4 times(*②)		*⑥
☐	Chromogen (TMB) (After dispense, the color turns to blue depending on the concentration.)	100 µL	
☐	↓Shaking(*③), Incubation for 30 minutes at 20-25 °C. (Standing(*④))		*⑧

□	Stop solution (1M H_2SO_4) After dispense, the color turns to yellow depending on the concentration.	100 µL	
□	↓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 seconds and remove the buffer. Guideline of washing volume: 300 µL/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: 5-25 mL/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1200 rpm for 10 seconds ×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 nm -650 nm can be used as reference wavelength.

*⑥After removal of wash buffer, immediately dispense the next reagent.

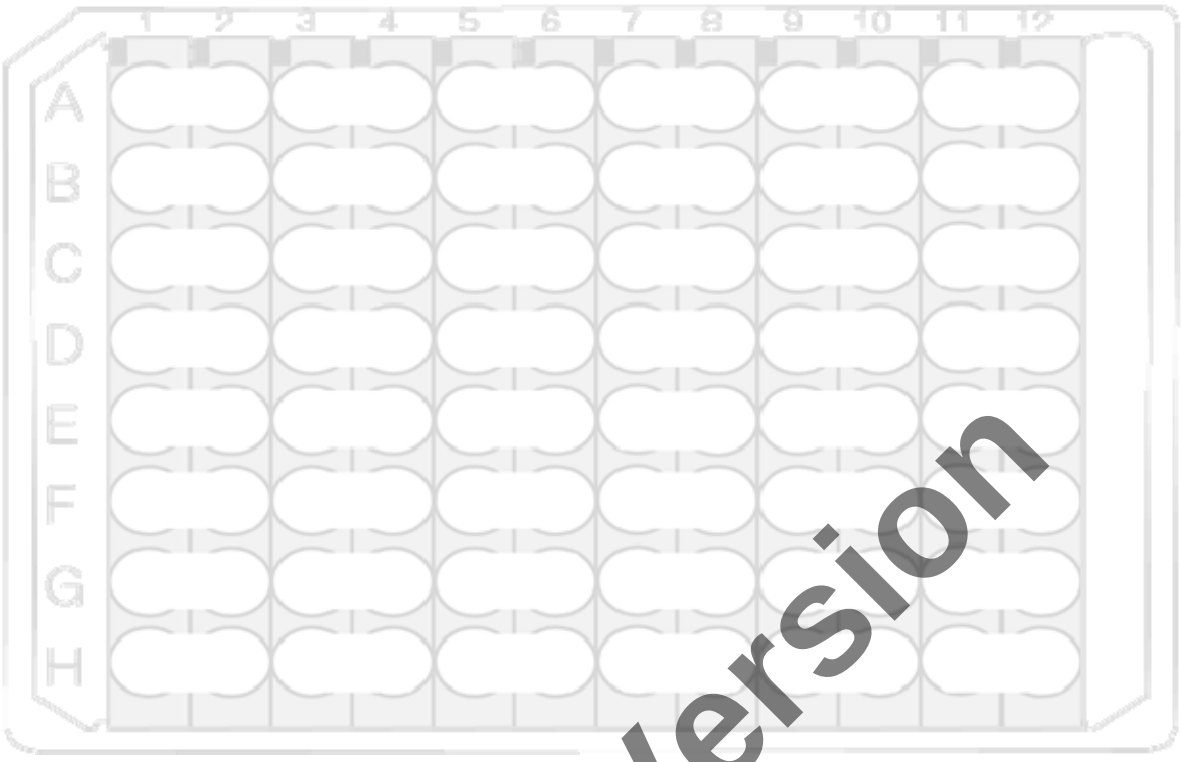
*⑦Refer to our web movie [Handling of pipetting].

*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 ng/mL	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	6.0 ng/mL	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	3.0 ng/mL	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	1.5 ng/mL	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	0.75 ng/mL	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	0.375 ng/mL	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	0.188 ng/mL	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	0(Blank)	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

Assay worksheet



LBIS Porcine Insulin ELISA Kit

[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.)

[Manufacture #] AKRIN-013T

[Cat #] 634-01469

This kit is manufactured by **FUJIFILM Wako Shibayagi Corporation**

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