For Research Use only

October 14, 2011 Ver. 2

[Mouse/Rat Proinsulin ELISA Kit]

(Code No.:AKMPI-111)

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/Rat Proinsulin ELISA Kit is a sandwich ELISA system for quantitative measurement of total mouse/rat proinsulin. 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 the label on the box. Opened reagents should be used as soon as possible to avoid losing its optimal assay performance caused by storage environment.

3. Introduction

Proinsulin, known as a precursor of insulin, is produced in B-cells of islet of Langerhans in the pancreas. Original single chain, with a molecular weight of 94KDa is divided into insulin and C-peptide after forming of three disulfide bonds and enzymatic splicing during the moving process to secretory granules. About 10% of proinsulin remains unsplitted and included in secretory granules. Biopotency of proinsulin is reported to be 5-10% of insulin. In usual immunoassay values of insulin and C-peptide (IR-insulin, IR-C-peptide), amount of proinsulin is also included. In general, the ratio of proinsulin to insulin reflects the state of processing of insulin biosynthesis. In NIDDM(DM2) and obesity plasma levels of proinsulin and its ratio to insulin increase compared with normal subject probably due to enhanced insulin secretion causes release of premature secretory granules. The ratio also increases in insulinoma, familial hyperproinsulinemia and hyperthyroidism.

4. Assay principle

In Shibayagi's Mouse/Rat Proinsulin ELISA Kit, standards or samples are incubated in monoclonal anti-proinsulin-coated wells to capture proinsulin After 2 hours incubation and washing, biotin-conjugated anti-proinsulin is added and incubated further for 2 hours to bind with captured proinsulin. After washing, HRP (horse radish peroxidase)-conjugated avidin is added, and incubate for 30 minutes. After washing, bound HRP-conjugated avidin is reacted with reacted with a substrate chromogen reagent (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 proinsulin concentration. The standard curve is prepared by plotting absorbance against standard proinsulin concentrations. Proinsulin 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 solution 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°C strictly. Avoid airstream velocity over 0.4 m/sec. (including wind from air conditioner), and humidity less than 30%. For more details, watch our web movie [Assay circumstance].

6. Reagents supplied

Components	State	Amount
(A) Anti-proinsulin-coated plate	Use after washing	60 wells*/1 plate
(B) Standard proinsulin solution (200 pmol/l) (derived from mouse pancreas extract)	Concentrated. Use after dilution	100 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin conjugated anti-proinsulin	Concentrated. Use after dilution.	100 µl/1 vial
(E) HRP conjugated streptavidin	Concentrated. Use after dilution.	100 µl/1 vial
(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 cover	−	1 plate
Instruction Manual	−	1 copy

*96 well-plate is provided, however, use the central 60 wells for your assay to avoid edge effect which promotes the reaction of exterior wells more than central wells.

7. Equipments or supplies 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 10-50 µl precisely, and another for 100-400 µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 µl.
☐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) Standard proinsulin solution (200 pmol/l)]

Make a serial dilution of master standard solution to prepare each standard solution.
Use the standards of 10, 2.5, 1.25, 0.625, and 0.156 pmol/L for the assay.

Volume of standard solution	Buffer solution	Concentration (pmol/l)	Concentration (pg/ml)
Original solution: 20 µl	380 µl	10	94.3
10 pmol/l solution: 200 µl	200 µl	5.0	47.2
5 pmol/l solution: 200 µl	200 µl	2.5	23.6
2.5 pmol/l solution: 200 µl	200 µl	1.25	11.8
1.25 pmol/l solution: 200 µl	200 µl	0.625	5.9
0.625 pmol/l solution: 200 µl	200 µl	0.313	2.95
0.313 pmol/l solution: 200 µl	200 µl	0.156	1.47
0 (Blank)	200 µl	0	0

[(D) Biotin conjugated anti-proinsulin]

Prepare working solution by dilution of (D) with the buffer solution (C) to 100x.
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 100x.
10 ml of the diluted solution is enough for 96 wells.

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

Dilute 1 volume of the washing buffer concentrate (10x) to 10 volume 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-proinsulin-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 proinsulin solution (200 pmol/l)]

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

[(C) Buffer solution] & [(F) Substrate chromogen reagent]

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.

[(D) Biotin conjugated anti-proinsulin] & [(E) HRP conjugated streptavidin]

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) Washing buffer concentrate (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 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.
- As the anti-insulin-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. For more details, watch our web movie [Assay circumstance].

10. Preparation of samples

This kit is intended to measure total mouse/rat proinlin in serum or plasma. Heparin (final conc. 1.2-12 U/ml) or EDTA-2Na (final conc. 1 mg/ml) is recommended for anticoagulant.

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. If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Sample dilution should be carried out with the buffer solution of the kit using small test tubes before assay. Mix well and pipette 50 µl of diluted sample (e.g. sample 10µl + buffer 40µl) into a well for assay. In the standard assay procedure, the dilution rate is 5x. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

Storage and stability

Proinsulin in samples will be inactivated if stored at 2-8°C. If it is necessary to store sample in refrigerator (2-8°C), 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 –35°C. 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-proinsulin-coated plate after bringing up to room temperature.

(1) Wash the anti-proinsulin-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 40µl of buffer and 10µl of sample to the designated sample wells (See worksheet example below).
(3) Pipette 50µl of standard solution to the wells designated for standards (See worksheet example below).
(4) Shake the plate gently on a plate shaker (*③).
(5) Put a plate cover on the plate and incubate for 2 hours at 20-25°C.
(6) Discard the reaction mixture, and then wash the plate as step (1).
(7) Pipette 50µl of biotin conjugated anti-proinsulin to all wells, and shake as step (4).
(8) Put a plate cover on the plate and incubate for 2 hours at 20-25°C.
(9) Discard the reaction mixture, and then wash the plate as step (1).
(10) Pipette 50 µl of HRP conjugated streptavidin to all wells, and shake as step (4).
(11) Put a plate cover on the plate and incubate the plate for 30 minutes at 20-25°C.
(12) Discard the reaction mixture, and then wash the plate as step (1).
(13) Pipette 50µl of substrate chromogen reagent to wells, and shake as step (4).
(14) Put a plate cover on the plate and incubate the plate for 30 minutes at 20-25°C.
(15) Add 50 µl of the reaction stopper to all wells and shake as step (4).
(16) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

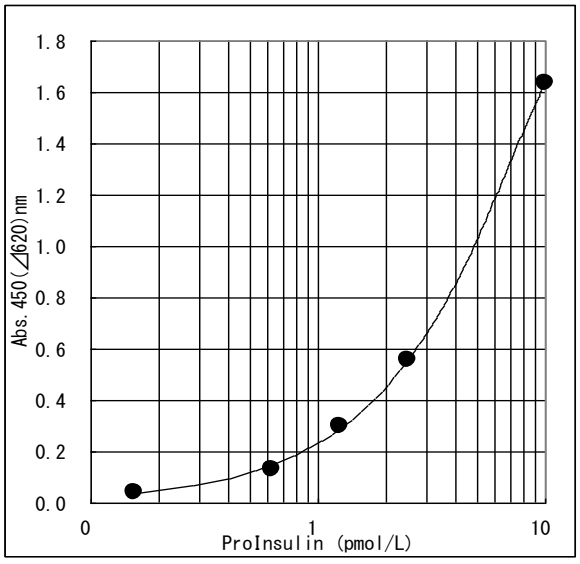
*Refer to the page 7-8 for notes of *② and *③.

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 proinsulin 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.

Mouse proinsulin assay standard curve (an example)

Absorbance may change due to assay environment.



13. Performance characteristics

● Assay range

The assay range of the kit is 0.156 ~ 10 pmol/l (1.47 ~ 94.3 pg/ml).
For 5x dilution, 0.78 ~ 50 pmol/l (7.35 ~ 471.5 pg/ml)

● Specificity

The antibodies used in this kit are specific to proinsulin. Cross-reactivity of the kit is shown below. *Cross-reactivity at Conc. 10 pmol/l

Substances	Cross-reactivity (%)
Mouse proinsulin	100
Mouse insulin/C-peptide/Leptin	-
Rat proinsulin	100
Rat insulin/C-peptide/Leptin	-
Human Proinsulin	+
Human Insulin/C-peptide	-

● Precision of assay

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

● Reproducibility

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

● Recovery test

Standard proinsulin was added in 3 concentrations to 2samples and assayed.
The recoveries were 92.5 ~109%

● Dilution test

Two samples were serially diluted by 3 steps.
The dilution curves showed linearity with R^2 close was 0.9994-0.999.

14. Reference assay data

Mouse strains: 5 BALB/c, 8 weeks, female and 10 ICR, 7 weeks, male mice, fed *ad libitum,* Serum.
Assay values (mean ± SD) BALB/c: 2.46 ± 0.598 pmol/l, ICR: 3.04 ± 1.37 pmol/l
Rat strain: 7 GK, 16 weeks, male, fed *ad libitum,* Serum
Assay values (mean ± SD): 3.31 ± 1.48 pmol/l
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-proinsulin, HRP conjugated streptavidin, or Substrate chromogen reagent might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin conjugated anti-pro insulin 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 substrate chromogen reagent soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

● Intense coloration in all wells including blank

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.)
2) Overdeveloping. Incubation time with substrate chromogen reagent should be decreased before addition of reaction stopper.
3) Too high incubation temperature. Adjust the temperature to 20-25°C.

● 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 ☐ : 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].

☐Bring the well-plate and all reagents back at 20~25 ºC for 2 hours.

☐Washing buffer concentrate must be diluted to 10 times by purified water that returned to 20~25 ºC .

☐Standard Proinsulin solution dilution example:

Concentration (pmol/l)	10	5.0	2.5	1.25	0.625	0.313	0.156	0
Std. proinsulin solution (µl)→ orig.sol.	20	200*	200*	200*	200*	200*	200	0
Buffer solution (µl)	380	200	200	200	200	200	200	200

*One rank higher standard.

☐Prepare the positive sample.

			Precautions & related info
☐	Anti-Proinsulin-coated plate		
☐	↓ Washing 4 times (*②)		*⑥
☐	Diluted samples/Standards	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking (*③), Incubation for 2 hours at 20-25oC. (Standing (*④))		*⑧ [Assay circumstance]
☐	Biotin conjugated anti-proinsulin(D) Dilute to 100x by using buffer solution(C)		Dilute reagents during the first reaction.
☐	↓ Washing 4 times (*②)		*⑥
☐	Biotin conjugated anti-proinsulin	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking (*③), Incubation for 2 hours at 20-25oC. (Standing (*④))		*⑧ [Assay circumstance]
	HRP conjugated streptavidin(E) Dilute to 100x by using buffer solution(C)		Dilute reagents during the second reaction.
☐	↓ Washing 4 times (*②)		*⑥
☐	HRP conjugated streptavidin	50 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)	50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking (*③), Incubation for 30 minutes at 20-25oC. (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	50 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.

*②Guideline 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.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.
*④Put a plate cover on the plate during the reaction after shaking.
*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 recommended

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		STD 10pmol/L	STD 10pmol/L	Positive control	Positive control	Sample 6	Sample 6	Sample 12	Sample 12	Sample 18	Sample 18	
C		STD 2.5pmol/L	STD 2.5pmol/L	Sample 1	Sample 1	Sample 7	Sample 7	Sample 13	Sample 13	Sample 19	Sample 19	
D		STD 1.25pmol/L	STD 1.25pmol/L	Sample 2	Sample 2	Sample 8	Sample 8	Sample 14	Sample 14	Sample 20	Sample 20	
E		STD 0.625pmol/L	STD 0.625pmol/L	Sample 3	Sample 3	Sample 9	Sample 9	Sample 15	Sample 15	Sample 21	Sample 21	
F		STD 0.156pmol/L	STD 156pmol/L	Sample 4	Sample 4	Sample 10	Sample 10	Sample 16	Sample 16	Sample 22	Sample 22	
G		Blank	Blank	Sample 5	Sample 5	Sample 11	Sample 11	Sample 17	Sample 17	Sample 23	Sample 23	
H												

*As this product is highly sensitive, above recommendation is made to avoid edge effect.

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^{o}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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