MK155

For Research Use

TaKaRa

Human/Mouse/Rat C-Peptide Competitive EIA Kit

Product Manual

v201608

Table of Contents

I. Description

C-peptide is processed from proinsulin that is produced in pancreatic β cells. C-peptide is a 31 amino acid peptide that is derived from the center of proinsulin, between the insulin B chain (30 amino acid residues) at the N-terminus and the insulin A chain (21 amino acid residues) at the C-terminus (Figure 1). One molecule of C-peptide corresponds to one insulin molecule by proinsulin processing.

This kit is a single antibody-antigen competitive EIA (enzyme immunoassay) used to quantify insulin C-peptide in serum, plasma, urine, and cell culture media. The kit uses a rabbit polyclonal antibody that reacts with C-peptide from human, mouse, and rat. The kit includes a plate pre-coated with an immobilized anti-rabbit secondary antibody that is used to capture the C-peptide specific antibody before sample addition. The kit uses an avidin-biotin system, allowing many samples to be measured easily with high sensitivity in about 5 hours.

The physiological function of C-Peptide is not clear. The half-life of C-Peptide is 2 - 5 times longer than that of insulin. It is thought that C-Peptide concentration is higher than insulin in the peripheral blood of healthy individuals, and that blood C-Peptide concentration reflects the production of proinsulin in the pancreas more accurately than insulin concentration.

C-peptide, unlike insulin, is excreted in the urine at levels 20 - 50 times higher than in blood. The average insulin production ability of pancreatic β cells can be monitored by measuring excretion of C-peptide in 24-hour urine collection.

Quantification of C-peptide can be used for:

a) Evaluation of pancreatic β cell function during insulin administration.
b) Classification of type I and II diabetes.
c) Evaluation of pancreatic function under hypoglycemia.
   Insulin administration can decrease blood glucose levels without increasing C-peptide concentration.
d) Detection and evaluation of insulinoma in animal models under external insulin administration.
e) Detection and evaluation of insulinoma after insulin inhibition test under normal blood glucose levels in animal models.
f) A marker of remaining pancreatic tissue function after experimental pancreatectomy; monitoring pancreas or β cell transplantation.

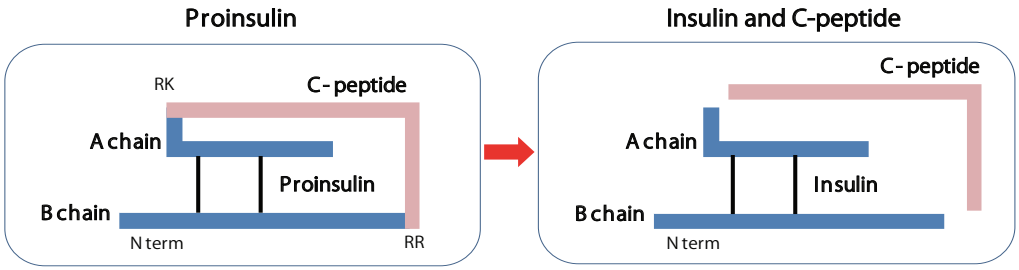


Figure 1. Insulin is produced from proinsulin.

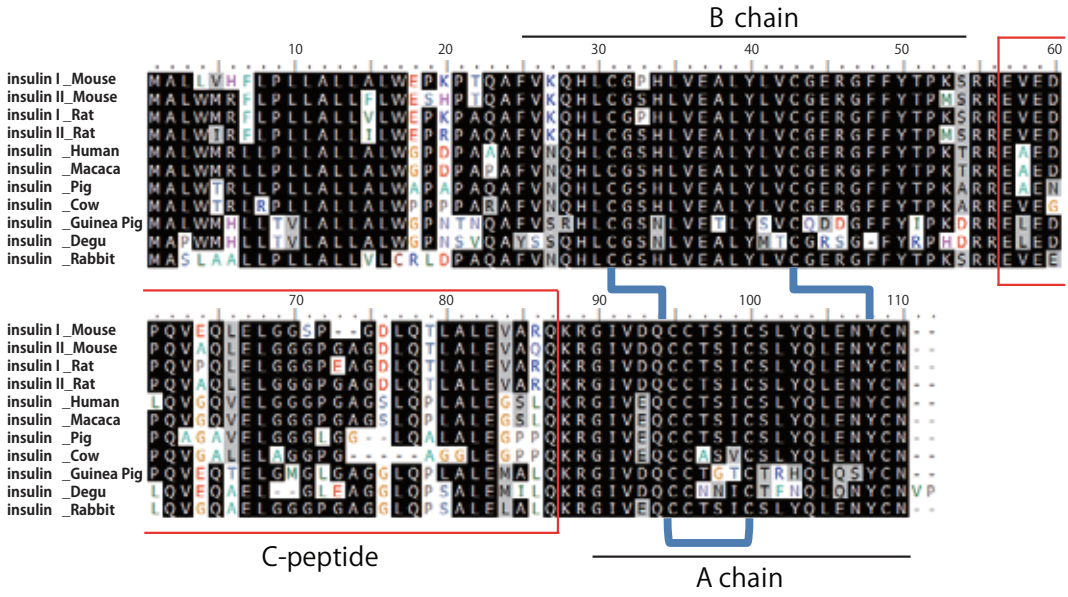
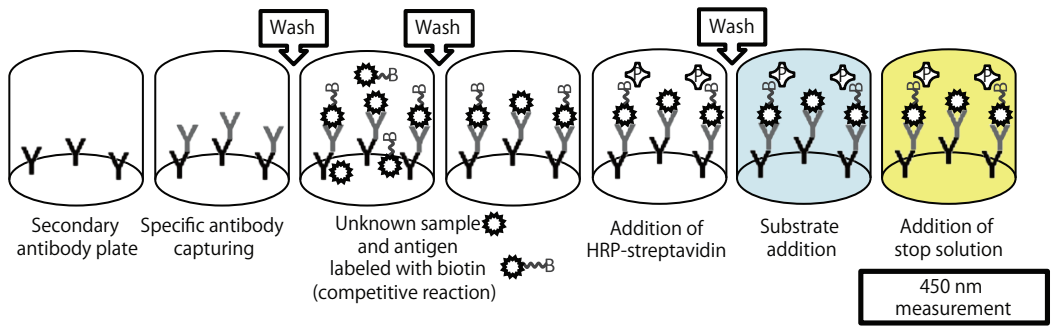


Figure 2. Amino acid sequence of preproinsulin. Proinsulin is produced when the signal peptide is separated from preproinsulin.

II. Principle

This kit detects antigen using a one antibody-antigen competitive EIA method. In this method, a 96 well solid phase plate coated with a secondary antibody is used to capture C-peptide specific antibody. Specifically, a C-peptide specific rabbit polyclonal antibody is captured by an anti-rabbit secondary antibody immobilized on the 96 well solid phase plate, and excess antibody is removed from wells by washing. Next, the test samples and standard solutions are prepared and mixed 1 : 1 with C-peptide labeled with biotin. Then the prepared samples are added to each well of the plate containing immobilized C-Peptide specific antibody. After the reaction, excess sample is removed by washing, and then streptavidin labeled with HRP is added to the wells. After the wells are washed once more, TMBZ substrate solution is added and a colometric reaction occurs. If the C-peptide concentration is high in a sample, less color develops because the amount of biotin labeled C-peptide in the well is diminished due to the competition. After the reaction stop solution is added, absorbance at 450 nm can be measured using a plate reader to determine C-peptide concentration.

This kit reacts with C-peptide specifically and does not cross-react with insulin A and B chains.



III. Components

(1)	Secondary Antibody Microtiterplate Secondary antibody coated plate (96 well : 8-well x 12 strips)	1 plate
(2)	Wash Buffer Concentrate (20X) 20 times concentrated wash solution	25 ml
(3)	Standard C-Peptide (100 μg/ml) Synthetic C-peptide	10 μl x 2
(4)	Anti C-Peptide PoAb Anti C-Peptide rabbit polyclonal antibody	5 μl x 2
(5)	Assay Diluent A Diluent for specimen (for serum or plasma) ＊ Containing 0.09% Sodium azide as a preservative	30 ml
(6)	Assay Diluent B (5X) Diluent for specimen (for culture supernatant or urine) and reagent preparation	15 ml
(7)	Biotinylated C-Peptide (10 μg/ml) C-Peptide labeled with biotin	20 μl x 2
(8)	HRP-Streptavidin Concentrate HRP-labeled streptavidin concentrate	0.6 ml
(9)	Positive Control	0.1 ml
(10)	Substrate Solution (TMBZ) 3,3′ ,5,5′ -tetramethylbenzidine solution	12 ml
(11)	Stop Solution without Sulfuric Acid Reaction stop solution (without sulfuric acid)	12 ml

IV. Materials Required but not Provided

- Microplate reader (that can measure absorbance at 450 nm)
- 96 well plates or microtubes
- Micropipettes and tips
- Multi-channel pipette
- Plate-washing instrument
  - Note 1: Personal Microplate Washer (Cat. #MK950)＊
  - Note 2: If there is insufficient wash buffer for use in an automated ELISA processing system, prepare 0.1% Tween 20/PBS using “Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)” and use as the wash buffer.
- Plate mixer
- Incubator (20 - 30℃)
- Pipettes for 1 - 25 ml (for reagent preparation)
- 100 ml and 1 L graduated cylinders
- Paper towels
- Distilled water
- Software to analyze ELISA data

＊ : Not available in all geographic locations. Check for availability in your area.

V. Storage

- Store unopened kit at -20℃.
  Avoid repeated freezing and thawing. Once thawed, store at 2 - 8℃ for ~6 months.

- After opening, microplate and reagents can be stored at 2 - 8℃ for ~1 month. Store any unused wells of the microplate in the provided pouch with desiccant.

VI. Intended Use

Measurement of C-peptide in serum, plasma, urine, and cell culture medium

＊ This product can be used for measurement of human, mouse, and rat C-peptide.

VII. Protocol

Bring all reagents and samples to room temperature (20 - 30℃) before use.

1. Preparation of reagents

- Secondary antibody plate [(1) Secondary Antibody Microtiterplate]
  Bring to room temperature before use and unseal.

- Wash solution
  Dilute 25 ml of (2) Wash Buffer Concentrate (20X) with 475 ml of distilled water, and mix well.
  ＊ If a precipitate is observed, bring to room temperature and mix gently to dissolve.
  ＊ When an automated ELISA processing system is used, there may be insufficient wash buffer. In this case, prepare 0.1% Tween 20/PBS using "Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)."

- Assay Diluent A (for dilution of serum and plasma)
  Use (5) Assay Diluent A directly. It contains 0.09% sodium azide as a preservative. When measuring C-peptide from serum or plasma, use to dilute standard solutions and samples.

- Assay Diluent B (for preparation of reagents and dilution of culture medium and urine)
  Dilute (6) Assay Diluent B (5X) 5 times with distilled water and to prepare 1X Assay Diluent B. When measuring C-peptide from cell culture medium or urine, use to dilute standard solutions and samples.

- C-Peptide standard solution [(3) Standard C-Peptide]
  Spin down briefly to ensure that all of the solution is brought to the bottom of the tube (10 μl). Add 490 μl of either Assay Diluent A or 1X Assay Diluent B (use the same diluent for sample dilution) and mix well using a pipette to prepare a 2,000 ng/ml C-Peptide standard solution. The C-Peptide standard solution (2,000 ng/ml) can be stored at -20℃ (-80℃ is recommended).
  Prepare a standard dilution series following VII-3.

- Anti C-Peptide rabbit polyclonal antibody [(4) Anti C-Peptide PoAb]
  Spin down briefly to ensure that all of the solution is brought to the bottom of the tube (5 μl). Add 50 μl of 1X Assay Diluent B into the vial and mix well using a pipette. This is the stock solution; the solution is diluted 100 times for use. One vial contains sufficient reagent for 48 wells (two vials are included with this kit).

- C-Peptide labeled with biotin [(7) Biotinylated C-Peptide (10 μg/ml)]
  Spin down briefly to ensure all of the solution is brought to the bottom of the tube (20 μl). Remove 12 μl and dilute 500 times with 6 ml of "1X Assay Diluent B" to prepare 20 ng/ml C-Peptide labeled with biotin. Two vials are included in this kit.

- HRP-labeled streptavidin [(8) HRP-Streptavidin Concentrate]
  Spin down briefly. Dilute 200 - fold with 1X Assay Diluent B.

  Preparation example:
  Mix gently by pipetting.
  Take 60 μl (amount adequate for 1 plate) and mix with 12 ml of 1X Assay Diluent B.
  Use the prepared streptavidin labeled with HRP within 24 hours. The diluted solution cannot be stored.

- Positive control [(9) Positive Control]
  A positive control is used to confirm successful measurement.
  When diluted 2 - fold using the same diluent as used for sample dilution, it can be detected in the 1 - 100 ng/ml range.

- Reaction stop solution [(11) Stop Solution without Sulfuric Acid]
  Use directly.
  ＊ After adding to wells, mix using a plate mixer etc. as the solution is highly viscous. The color reaction is stable for 6 hours.

2. Samples

- Use (5) Assay Diluent A for dilution of serum or plasma samples.
- Prepare 1X Assay Diluent B and use for dilution of urine or cell culture medium samples＊.
- For standard solutions, measurement accuracy can be improved by using the same diluent as used for sample dilution.

Standard dilution range for normal serum and plasma : 2 - 4 fold dilution
Standard dilution range for urine : 2 - 16 fold dilution

Because the amount of C-peptide differs depending on the sample, determine the optimal dilution ratio experimentally.

＊: Although the amount of bovine antigen in cell culture medium is below the level of detection in most cases, the amount may vary between different lots of fetal bovine serum. When a sample containing 10% fetal bovine serum is measured using this kit, perform a control experiment with media only.

3. Preparation of standard dilution for a standard curve

Prepare standard solutions (1,000, 100, 10, 1, and 0.1 ng/ml) with the same diluent as used for sample dilution. Dilute the prepared C-Peptide standard solution (2,000 ng/ml) 2 - fold (1,000 ng/ml standard). Prepare the other standards by 10 - fold serial dilution.
Mix well at each dilution step. Changing the pipette tip will improve accuracy.
Also prepare one 0 ng/ml standard using with diluent only (Assay Diluent A or 1X Assay Diluent B).

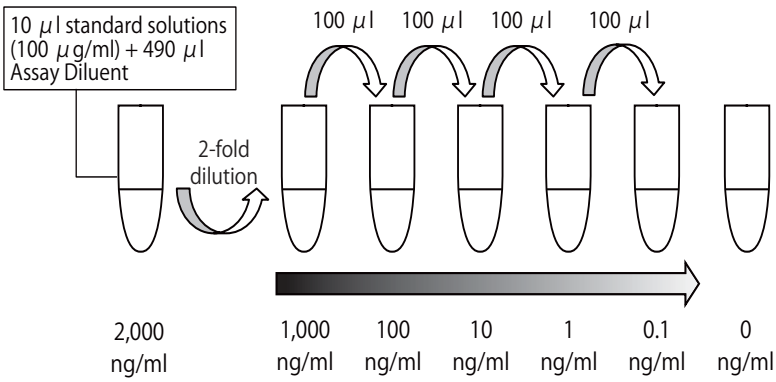


Figure 3. Preparing the standard solutions.

4. Procedure

Bring reagents and samples to room temperature (20 - 30℃) before use. Measure standard solutions and samples in at least duplicate.

1. Dilute the prepared stock solution of anti-C-Peptide rabbit polyclonal antibody 100 - fold with 1X Assay Diluent B, and dispense 100 μl per well of the Secondary Antibody Microtiterplate. Allow the plate to stand at room temperature for 1.5 hours. If measurement will not be performed on the same day, store the plate overnight at 4℃.

2. Remove the antibody solution and wash 4 times with 300 μl wash solution. Use a plate-washing instrument or a multi-channel pipette to add the wash solution to each well. It is important to remove reaction mixture completely at each step. After washing, remove the wash solution well by aspiration (suction) or by decanting. Further, turn the plate upside down and then hit it against a paper towel on a hard surface several times to drain the liquid.

3. Add 50 μl of each concentration of standard solution and diluted samples into another 96 well plate or microtubes, add 50 μl of the prepared C-Peptide labeled with biotin (20 ng/ml) into each well or tube, and mix well.

4. Add all of the mixture＊ prepared in step 3 (100 μl) into the plate with specific antibody from step 2. Incubate for 2.5 hours at room temperature.
   ＊ : If the amount is <100 μl, adjust to input the same amount for all samples and standards.

5. Remove the reaction mixture and wash 4 times with wash solution following the procedures in step 2.

6. Add 100 μl of the prepared streptavidin labeled with HRP to each well. Incubate for 45 minutes at room temperature.

7. Remove the reaction solution and wash 4 times with wash solution following the procedures in step 2.

8. Add 100 μl Substrate Solution (TMBZ) in each well.
   Allow the color reaction progress for 15 minutes at room temperature in the dark.

9. Add 100 μl of Reaction Stop Solution to each well in the same order that the Substrate Solution was added, and mix well using a plate mixer.

10. Measure absorbance at 450 nm using a plate reader.

[Summary of measurement method]

1. Prepare reagents.
   Prepare sample and standard solutions.

⇩

2. Add 100 μl of anti-C-Peptide antibody to each well of the Secondary Antibody Microtiterplate, incubate for 1.5 hours at room temperature or overnight at 4℃. — First reaction

⇩ Wash

3. Mix 50 μl of each standard solution/sample with C-Peptide labeled with biotin 1 : 1 (by volume). Add all of the mixture to the plate with specific antibody. Incubate for 2.5 hours at room temperature. — Second reaction

⇩ Wash

4. Add 100 μl of HRP-labeled streptavidin to each well.
   Incubate for 45 minutes at room temperature. — Third reaction

⇩ Wash

5. Add 100 μl of TMBZ to each well.
   Incubate for 15 minutes at room temperature.

⇩

6. Add 100 μl of Stop solution to each well.
   Measure absorbance at 450 nm.

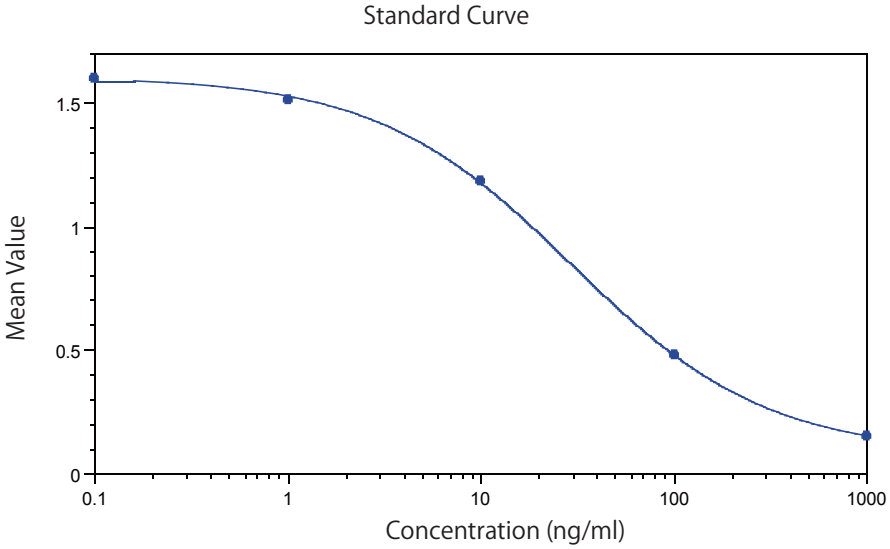
5. Calculation

A standard curve is prepared by plotting the results of standard solutions from duplicate measurements, with concentration as the x-axis and absorbance as the y-axis. Based on the absorbance value of the control and sample, the sample concentration is calculated from the standard curve. Since this system is a competitive ELISA method using one antibody, the absorbance shows the highest value when the concentration of the standard solution is 0, and the absorbance values decrease with increasing amounts of C-peptide in each sample.

VIII. Performance

1. Standard Curve

The following standard curve is a representative example. Prepare a standard curve for each experiment.



4-P Fit : $y = (A - D)/(1 + (x/C)^B) + D$:

A	B	C	D	R^2
1.6	0.857	30.1	0.0812	1

C-Peptide concentration (ng/ml)	1,000	100	10	1	0.1	0
A_{450}	0.155	0.479	1.183	1.512	1.602	1.683

2. Measurement range and sensitivity

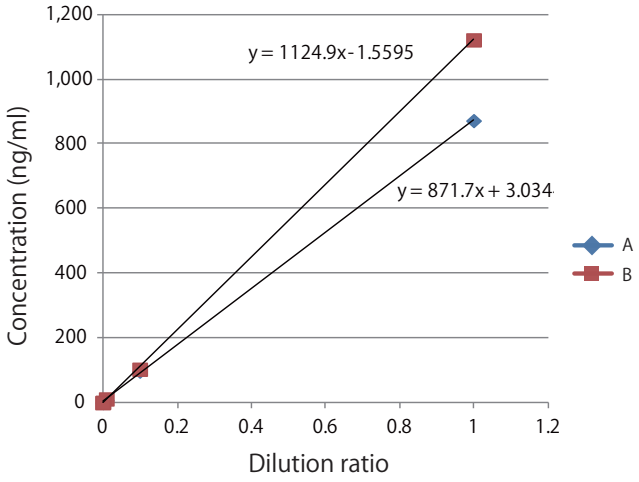
Measurement range: 0.1 - 1,000 ng/ml
Confidence range: 1 - 1,000 ng/ml
Minimum sensitivity: 0.8 ng/ml

3. Reproducibility

Intra-day (repeatability): CV<10%
Inter-day (reproducibility): CV<15%

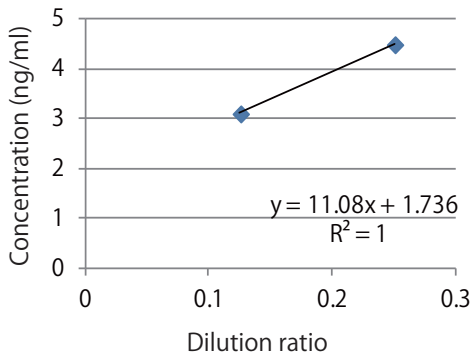
4. Linearity of Dilution

<Two highly concentrated peptide samples diluted with Assay Diluent A>



Dilution		Concentration (ng/ml)	
		A	B
1X	(1)	874	1,124
10X	(0.1)	98	104
100X	(0.01)	7.9	12
1,000X	(0.001)	0.8	1.7
10,000X	(0.0001)	-	0.37

<Mouse urine diluted with 1x Assay Diluent B>



Dilution		Concentration (ng/ml)
4X	(0.25)	4.5
8X	(0.125)	3.1

5. Specificity

The antibody used in this kit does not cross-react with:
Insulin-A chain, Insulin-B chain, Ghrelin, Nesfatin, Angiotensin II, NPY, APC

IX. References

1) Marques RG, Fontaine MJ, and Rogers J. C-Peptide: much more than a byproduct of insulin biosynthesis. *Pancreas.* (2004) **29**(3):231–8.
2) Wahren J, Ekberg K, Samnegård B, and Johansson BL. C-Peptide: a new potential in the treatment of diabetic nephropathy. *Curr Diab Rep.* (2001) **1**(3):261–6.
3) Wahren J. C-Peptide: new findings and therapeutic implications in diabetes. *Clin Physiol Funct Imaging.* (2004) **24**(4):180–9.