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CRYSTAL CHEM INC.

Rat Insulin ELISA Kit Instructions

For the quantitative determination of insulin
in rat serum, plasma, and fluid

Catalog #90010

96 Assays

For research use only. Not for use in diagnostic procedures.

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A. Intended Use

The Rat Insulin ELISA kit is for the quantitative determination of insulin in rat serum, plasma, and fluid. Please read the complete kit insert before performing this assay. The kit is for *RESEARCH USE ONLY*. It is not intended for use in clinical or diagnostic procedures or for internal or external use in humans or animals.

B. Introduction

Insulin is the primary hormone produced in the ß cells of the islets of Langerhans, and is known not only to regulate glucose metabolism, *i.e.* the uptake of blood glucose to the liver and peripheral tissues, but also play other important physiological roles.

Recent increases in the incidence of diabetes and obesity have stimulated intensive research on insulin levels and production. As a result, the accurate measurement of insulin in experimental animals is becoming increasingly important.

The kit is a simple, precise, and sensitive ELISA sandwich assay for rat insulin. The following assays can be run using the Rat Insulin ELISA kit:

TABLE 1 Sensitivity range of assay

Assay	Sensitivity Range (based on 5 µL sample)
Serum, Plasma and Fluid	0.156 – 10 ng/mL*

* An increased sensitivity of 39 pg/mL can be achieved using a 20 µL sample.

C. Principles of the Assay

1. First reaction

Insulin in the sample is bound to the mouse anti-insulin monoclonal antibody coated on a microplate well and the anti-insulin antibody of the guinea pig serum is added to each well. Consequently the mouse anti-insulin monoclonal antibody /insulin/guinea pig anti-insulin antibody complex is immobilized to the microplate well.

2. Washing

Unbound material is removed by washing.

3. Second reaction

Horse radish peroxidase (POD)-conjugated anti-guinea pig antibody is then bound to the guinea pig anti-insulin antibody complex immobilized to the microplate well.

4. Washing

Excess POD-conjugate is removed by washing.

5. Enzyme reaction

The bound POD conjugate in the microplate well is detected by the addition of the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution.

6. Measurement of absorbance

7. Evaluation of results

The insulin concentration is determined via interpolation using the standard curve generated by plotting absorbance versus the corresponding concentration of rat insulin standard.

D. Kit Storage

1. Upon receipt of the Rat Insulin ELISA kit, store it at 2-8˚C and avoid light exposure (do not freeze the kit or hold it at temperatures above 25˚C).
2. The kit must not be used after the expiration date.

E. Assay Materials

E. 1. Materials supplied

TABLE 2 Contents of the kit

Mark	Description	Amount
A	Antibody-coated Microplate (One pack contains 6 x 8 well modules, *i.e.* 48 wells / pack)	2 packs
B	Rat Insulin Standard, Lyophilized	2 ng/vial (for 200 µL)
C	Guinea Pig Anti-Insulin, Lyophilized Serum	2 vials (for 6 mL each)
D	Anti-Guinea Pig Antibody Enzyme Conjugate, Lyophilized	2 vials (for 6 mL each)
E	Enzyme Substrate (TMB) Solution	1 bottle (13 mL)
F	Enzyme Reaction Stop Solution (1 N Sulfuric Acid)	1 bottle (13 mL)
G	Sample Diluent	1 bottle (30 mL)
H	Wash Buffer Stock Solution (20X Concentrate)	1 bottle (50 mL)
	Frame for affixing the microplate well module	1 piece
	Plastic microplate cover	1 piece

E. 2. Materials required but not provided

Micropipettes and disposable tips
Volumetric pipettes and volumetric cylinders
Distilled or deionized water
Polypropylene test tubes
Test tube racks
Vortex mixer
Aspirator for washing procedure
Microplate reader (capable of measuring A_{450} and subtracting A_{630} values)

F. Reagent Precautions

1. Avoid direct contact with the Enzyme Substrate Solution (marked “E”) and the Enzyme Reaction Stop Solution (marked “F”). In case of contact, flush eyes or skin with plenty of water and get medical advice.
2. Do not allow the Enzyme Substrate Solution (marked “E”) to contact any metal.
3. Only appropriately-trained personnel should use the kit. Laboratory personnel should wear suitable protective clothing. All chemicals should be considered potentially hazardous.

G. Maximizing Kit Performance

1. Given the small sample volumes required (5 µL), pipetting should be done as carefully as possible. A high quality 10 µL or better precision pipette should be used for such volumes. Drops of liquid adhering to the outside of the pipette tips should be removed by wiping to ensure the highest degree of accuracy.
2. In order to prevent the microplate wells from drying out, samples and reagents should be dispensed quickly into the wells. In no case should 10 minutes be exceeded per plate per pippeting step.
3. The wash procedure should be done thoroughly in order to minimize background readings.
4. Each standard and sample should be assayed in duplicate.
5. The same sequence of pipetting and other operations should be maintained in all procedures.
6. Do not mix reagents that have different lot numbers.

H. Preparation of Rat Plasma and Serum

Plasma : Collect blood into a tube containing an anticoagulant such as heparin (final concentration: 1 unit/mL), EDTA (final concentration: 0.1%), or sodium citrate (final concentration: 0.76%), and centrifuge at 4°C for 20 min at 2,000 x *g*.

Serum : Collect blood, allow to clot, and centrifuge at 4°C for 20 min at 2,000 x*g*.

***Note** : Be sure to avoid hemolysis during*

preparation. Do not use turbid serum or plasma samples. Turbid serum or plasma should be centrifuged to produce a clear solution. Samples which need to be diluted must be diluted using the Sample Diluent (marked "G").

I. Assay Procedure (0.156 - 10ng/mL)

I. 1. Preparation of reagents

Prior to use, all reagents should be brought to room temperature (18-25°C), and should be stored at 2-8°C immediately after use. Before use, mix the reagents thoroughly by gentle agitation or swirling.

1. Antibody-coated microplate

   Remove the "Antibody-coated Microplate" (marked "A") from the sealed foil pouch after the pouch has been equilibrated to room temperature.

   ***Note** : The microplate must be used the same day as the pouch is opened.*

2. Rat insulin stock solution

   Reconstitute the "Rat Insulin Standard, Lyophilized" (marked "B") by careful addition of 200 µL of sample diluent to the vial. Invert the vial gently until the contents are completely dissolved. This stock solution contains 10 ng/mL of rat insulin. The reconstituted rat insulin stock solution is stable for one week at 2-8°C and one month at –20°C (avoid repeated freezing and thawing).

3. Guinea pig anti-insulin

   For six modules, prepare the needed volume of guinea pig anti-insulin solution by mixing 1 vial of "Guinea Pig Anti-Insulin, Lyophilized Serum" (marked "C") with 6 mL of the Sample Diluent (marked "G"), and mix completely to ensure a homogeneous solution.

   ***Note** : Guinea pig anti-insulin must be used immediately after reconstitution.*

4. Anti-guinea pig antibody enzyme conjugate

   For six modules, prepare the needed volume of anti-guinea pig antibody enzyme conjugate solution by mixing 1 vial of "Anti-Guinea Pig Antibody Enzyme Conjugate,

Lyophilized" (marked "D") with 6 mL of the Sample Diluent (marked “G”), and mix completely to ensure a homogeneous solution.

***Note** : Anti-guinea pig antibody enzyme conjugate is used on the second day of the assay. Anti-guinea pig antibody enzyme conjugate must be used immediately after reconstitution.*

5. Enzyme substrate solution
   The “Enzyme Substrate Solution” (marked “E”) is provided as a ready-to-use preparation. Once the bottle is opened, the enzyme substrate solution is stable for one week at 2-8°C.
   ***Note** : Avoid exposure of the enzyme substrate solution to light.*
6. Enzyme reaction stop solution (1N sulfuric acid)
   The "Enzyme Reaction Stop Solution" (marked "F") is provided as a ready-to-use preparation.
7. Sample diluent
   The “Sample Diluent” (marked “G”) is provided as a ready-to-use preparation. Once the bottle is opened, the sample diluent is stable for one week at 2-8°C.
8. Wash buffer
   The “Wash Buffer Stock Solution” (marked “H”) should be brought to 1 L with distilled or deionized water in a volumetric flask. Mix the solution well before use. The wash buffer is stable for one week at 2-8°C.

I. 2. Preparation of working rat insulin standards

1. Pipette 50 µL of Sample Diluent (marked “G”) and 50 µL of rat insulin stock solution (10 ng/mL) into a polypropylene microtube labeled 5 ng/mL, and mix thoroughly.
2. Dispense 50 µL of sample diluent into five polypropylene microtubes labeled 0.156, 0.313, 0.625, 1.25 and 2.5 ng/mL, respectively.
3. Dispense 50 µL of the 5 ng/mL standard into the 2.5 ng/mL microtube, and mix thoroughly.
4. Dispense 50 µL of 2.5 ng/mL standard into 1.25 ng/mL microtube, and mix thoroughly.
5. Repeat this dilution scheme using the remaining microtubes.

6. Dispense 50 µL of sample diluent into one polypropylene microtube labeled 0 ng/mL.

***Note** : The working insulin standards should be prepared shortly before use and discarded after use. Prepare working insulin standards using polypropylene microtubes because polypropylene exhibits minimal adsorption of insulin.*

TABLE 3 Preparation of working rat insulin standards

	Rat Insulin concentration (ng/mL)							
	10	5	2.5	1.25	0.625	0.313	0.156	0
RISS*(µL)	200	50						
SD**(µL)		50	50	50	50	50	50	50
			50 ↗	50 ↗	50 ↗	50 ↗	50	
Total (µL)	200	100	100	100	100	100	100	50

RISS* : Rat Insulin Stock Solution (10 ng/mL)
SD** : Sample Diluent

I. 3. Assay procedure

First reaction:

1. Remove the Antibody-coated Microplate modules (marked "A") from the sealed foil pouch after the pouch has been equilibrated to room temperature. Affix the microplate modules to the supporting frame.
2. In each well, dispense 95 µL of guinea pig anti-Insulin (reconstituted by sample diluent).
3. Pipette 5 µL samples (or 0, 0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 ng/mL working rat insulin standards) into the wells.

***Note** : Each standard and sample should be assayed in duplicate. It is also recommended that a 10 µL or better precision pipette be used when dispensing small volumes (5 µL).*

4. Cover the microplate with the plastic microplate cover and incubate overnight (16-20 hours) at 4˚C.

Second reaction:

5. Aspirate well contents and wash three times using 300 µL of wash buffer per well. After each wash, remove any remaining solution by inverting and tapping the plate firmly on a clean paper towel.

6. Dispense 100 µL per well of anti-guinea pig antibody enzyme conjugate (reconstituted by sample diluent).
7. Cover the microplate with the plastic microplate cover and incubate for 1 hour at room temperature.

Third reaction:

8. Aspirate well contents and wash five times using 300 µL of wash buffer per well. After each wash, remove any remaining solution by inverting and tapping the plate firmly on a clean paper towel.
9. Immediately dispense 100 µL per well of Enzyme Substrate Solution (marked “E”) and react for 30 minutes at room temperature. During the enzyme reaction, avoid exposing the microplate to light.

   ***Note** : Do not cover the microplate with aluminum foil.*
10. Stop the enzyme reaction by adding 100 µL per well of Enzyme Reaction Stop Solution (marked "F").
11. Measure absorbance within 30 minutes using a plate reader. (Measure A_{450} values and subtract A_{630} values)

I. 4. Determining the insulin concentration

1. Determine the mean absorbance for each set of duplicate standards or samples.

   ***Note** : If individual absorbance values differ from the mean by greater than 20%, performing the assay again is recommended. The mean absorbance of the 0 ng/mL standard should be less than 0.1.*
2. Using semi-log section paper, construct the insulin standard curve by plotting the mean absorbance value for each standard on the Y axis (linear) versus the corresponding standard rat insulin concentration on the X axis (logarithmic). Figure 1 is an example of a typical standard curve.

   ***Note** : A standard curve should be plotted every time the assay is performed. For computer data processing, a 4-Parameter curve, 5-parameter curve or Log/Logit curve is recommended.*
3. Rat insulin concentrations in the samples are interpolated using the standard curve and mean absorbance values for each sample.

***Note** : Samples with a high insulin concentration (10 ng/mL or higher) should be diluted with sample diluent and rerun.*

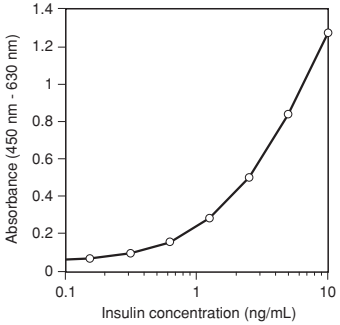

Figure 1 : A typical standard curve.

J. Appendix

J. 1. Performance characteristics

1. Precision : The intra-assay precision: C.V. ≦ 10%
   The inter-assay precision: C.V. ≦ 10%
2. Recovery : When rat insulin was spiked in a 5 µL rat plasma sample, the recovery was 100%± 10%.

J. 2. Increasing sensitivity

In cases in which samples are believed to contain an insulin concentration lower than 0.156 ng/mL (*i.e.* the lowest standard), the sample volume can be increased from 5 µL to a maximum of 20 µL to provide increased sensitivity. Using a 20 µL sample, the assay can detect a minimum insulin concentration of 39 pg/mL.

In such cases, the amount of guinea pig anti-insulin (reconstituted by sample diluent) added in Step 2 of Section I.3. should be decreased proportionately to the increase in sample volume to maintain a total reaction mixture of 100 µL in each well. As illustrated in Table 4, the more sample volume used, the less guinea pig anti-insulin should be added.

However, the volume of rat insulin standard (5 µL) should not be changed, even if the sample volume is greater than 5 µL. To account for the reduction of guinea pig anti-insulin added to each well, Sample Diluent should be added to all wells with standards proportionately to the increase in sample volume in order to maintain a total reaction mixture of 100 µL. Please see Table 4 below for additional reference:

TABLE 4 Increasing sensitivity through sample volume

Sample Volume	Wells for	Guinea Pig Anti-Insulin	Diluent	Standard Insulin	Sample	Total
5µL	Standard	95µL	—	5µL	—	100µL
	Sample	95µL	—	—	5µL	100µL
10µL	Standard	90µL	5µL	5µL		100µL
	Sample	90µL	—	—	10µL	100µL
15µL	Standard	85µL	10µL	5µL		100µL
	Sample	85µL	—	—	15µL	100µL
20µL	Standard	80µL	15µL	5µL		100µL
	Sample	80µL	—	—	20µL	100µL

After making the necessary adjustments indicated in Table 4, run the rest of the assay as indicated in Section I.3. The insulin concentration of the sample should be calculated as follows:

$$\text{Insulin concentration of sample (ng/mL)} = \text{Concentration obtained from standard curve (ng/mL)} \times \frac{5}{\text{Sample volume(µL)}}$$

J.3. Summary of reagent preparation

TABLE 5 Summary of reagent preparation

Reagent	Preparation Procedure
A: Antibody-coated Microplate	Ready to use
B: Rat Insulin Standard, Lyophilized	Dissolve in 200 µL of Sample Diluent
C: Guinea Pig Anti-Insulin, Lyophilized serum	For six modules* Reagent C - 1 vial Reagent G - 6 mL
D: Anti-Guinea Pig Antibody Enzyme Conjugate, Lyophilized	For six modules** Reagent D - 1 vial Reagent G - 6 mL
E: Enzyme Substrate (TMB) Solution	Ready to use
F: Enzyme Reaction Stop Solution (1 N Sulfuric Acid)	Ready to use
G: Sample Diluent	Ready to use
H: Wash Buffer Stock Solution (20X Concentrate)	Bring contents of the bottle to 1L with water***

***Note** : All reagents should be brought to room temperature (18-25°C) prior to use.*

* Prepare immediately before use.

** Prepare the second day immediately before use.

*** Distilled or deionized water.

J. 4. Summary of Rat Insulin ELISA kit assay

Affix the antibody-coated microplate (marked “A”) to the frame

⬇

Dispense 95 µL of guinea pig anti-insulin per well

⬇

Pipette 5µL of the sample (or working rat insulin standard) per well

⬇

Incubate the microplate overnight at 4˚C

⬇

Wash each well three times with wash buffer*

⬇

Dispense 100 µL of anti-guinea pig antibody enzyme conjugate per well

⬇

Incubate the microplate for 1 hour at room temperature

⬇

Wash each well five times with wash buffer*

⬇

Dispense 100 µL of Enzyme Substrate Solution (marked “E”) per well

⬇

Incubate microplate for 30 min at room temperature while avoiding exposure to light

⬇

Stop the reaction by adding 100 µL of Enzyme Reaction Stop Solution (marked “F”) per well

⬇

Measure A_{450} and subtract A_{630} values within 30 min

⬇

Calculate insulin concentrations using the standard curve

* Each well should be washed with 300 µL of wash buffer. Aspirate the wells completely so all excess solution is removed.

J.5. Assaying mouse insulin

Mouse insulin can also be assayed using the “Rat Insulin ELISA Kit”. For determining mouse insulin concentrations, we recommend using the optional mouse insulin standard (Catalog#90020).

***Note** : Each laboratory should establish its own expected range of values.*

Warranty

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