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CRYSTAL CHEM INC.

Ultra Sensitive Rat Insulin ELISA Kit Instructions

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

Catalog #90060

96 Assays

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

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A. Intended Use

The Ultra Sensitive 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 Ultra Sensitive Rat Insulin ELISA kit:

TABLE 1 Sensitivity range of assay

Assay	Sensitivity Range (based on 5 µL sample)
Low range assay (Section I)	0.1 – 6.4 ng/mL*
Wide range assay (Section J)	0.1 – 12.8 ng/mL**
High range assay (Section K)	1 – 64 ng/mL

* An ultra-high sensitivity of 5 pg/mL can be achieved using a 100 µL sample.

** Intended for screening purposes.

C. Principles of the Assay

1. **First reaction**
   Rat insulin in the sample is bound to the guinea pig anti-insulin antibody coated on the microplate well.
2. **Washing**
   Unbound material is removed by washing.
3. **Second reaction**
   Horse radish peroxidase (POD)-conjugated anti-insulin antibody is then bound to the guinea pig anti-insulin antibody/rat insulin 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 Ultra Sensitive 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 should 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 6x8 well modules, *i.e.* 48 wells / pack)	2 packs
B	Rat Insulin Standard, Lyophilized	2.56 ng/vial (for 100 μL)
C	Anti-Insulin Enzyme Conjugate Stock Solution	1 bottle (8 mL)
D	Enzyme Conjugate Diluent	1 bottle (4 mL)
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 flasks
Distilled or deionized water
Polypropylene microtubes
Test tube racks
Vortex mixer
Aspirator for washing procedure
Microplate reader (capable of measuring A_{450} and 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, immediately 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 pipetting 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. Low Range Assay (0.1 – 6.4 ng/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 100 µL of distilled or deionized water to the vial. Invert the vial gently until the contents are completely dissolved. This stock solution contains 25.6 ng/mL of rat insulin. The reconstituted rat insulin stock solution is stable for one week at 2-8°C.

3. Anti-insulin enzyme conjugate

   For six modules, prepare the needed volume of anti-insulin enzyme conjugate solution by mixing 3.6 mL of “Anti-Insulin Enzyme Conjugate Stock Solution” (marked “C”) with 1.8 mL of “Enzyme Conjugate Diluent” (marked “D”), and mix completely to ensure a homogeneous and clear solution. Avoid foaming during mixing.

   ***Note**: The anti-insulin enzyme conjugate should be prepared just before the second reaction and must be used immediately.*

4. 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.*

5. Enzyme reaction stop solution (1 N sulfuric acid)

   The “Enzyme Reaction Stop Solution” (marked “F”) is provided as a ready-to-use preparation.

6. 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.

7. 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 150 µL of sample diluent (marked "G") and 50 µL of rat insulin stock solution (25.6 ng/mL) into a polypropylene microtube labeled 6.4 ng/mL, and mix thoroughly.
2. Dispense 50 µL of sample diluent into six polypropylene microtubes labeled 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 ng/mL, respectively.
3. Dispense 50 µL of the 6.4 ng/mL standard into the 3.2 ng/mL microtube, and mix thoroughly.
4. Dispense 50 µL of the 3.2 ng/mL standard into the 1.6 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 (low range assay)

	Rat insulin concentration (ng/mL)							
	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0
RISS*(µL)	50							
SD**(µL)	150	50	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 (25.6 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 microplates to the supporting frame.
2. In each well, dispense 95 µL of sample diluent (marked "G").
3. Pipette 5 µL samples (or 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 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 for 2 hours at 4°C.

Second reaction:

5. 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.
6. Dispense 100 µL per well of anti-insulin enzyme conjugate.
7. Cover the microplate with the plastic microplate cover and incubate for 30 minutes at room temperature.

Third reaction:

8. Aspirate well contents and wash seven 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 and react for 40 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.*

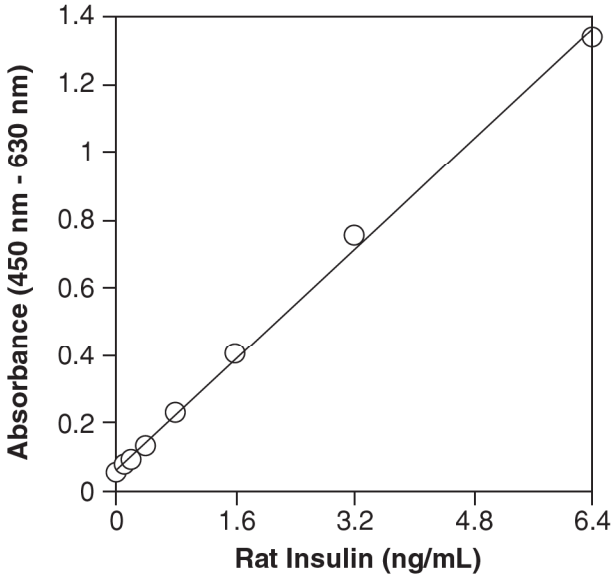
2. Using linear graph paper, construct the insulin standard curve by plotting the mean absorbance value for each standard on the Y axis versus the corresponding standard rat insulin concentration on the X axis. Figure 1 is an example of a typical standard curve generated by the low range assay.

   ***Note**: A standard curve should be plotted every time the assay is performed.*

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 (6.4 ng/mL or higher) should be diluted with the sample diluent and rerun.*

Figure 1 A typical low range standard curve (linear fit)



J. Wide Range Assay (0.1 – 12.8 ng/mL)

***Note**: This assay procedure is intended for screening purposes. It is recommended that samples with a reading of 6.4 ng/mL or higher be diluted and rerun using the low range assay in order to obtain accurate values.*

J.1. Preparation of reagents

1. Prepare all the reagents for use according to Section I.1 under Low Range Assay.

J.2. Preparation of working rat insulin standards

1. Pipette 50 µL of sample diluent (marked "G") and 50 µL of rat insulin stock solution (25.6 ng/mL) into a polypropylene microtube labeled 12.8 ng/mL, and mix thoroughly.
2. Dispense 50 µL of sample diluent into seven polypropylene microtubes labeled 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng/mL, respectively.
3. Dispense 50 µL of the 12.8 ng/mL standard into the 6.4 ng/mL microtube, and mix thoroughly.
4. Dispense 50 µL of the 6.4 ng/mL standard into the 3.2 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 4 Preparation of working rat insulin standards (wide range assay)

	Rat Insulin concentration (ng/mL)								
	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0
RISS*(µL)	50								
SD**(µL)	50	50	50	50	50	50	50	50	50
		50	50	50	50	50	50	50	
		↗	↗	↗	↗	↗	↗	↗	
Total (µL)	100	100	100	100	100	100	100	100	50

RISS* : Rat Insulin Stock Solution (25.6 ng/mL)
SD** : Sample Diluent

J.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 microplates to the supporting frame.
2. In each well, dispense 95 µL of sample diluent (marked "G").
3. Pipette 5 µL samples (or 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 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 for 2 hours at 4°C.

Second reaction:

5. 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.
6. Dispense 100 µL per well of anti-insulin enzyme conjugate.
7. Cover the microplate with the plastic microplate cover and incubate for 30 minutes at room temperature.

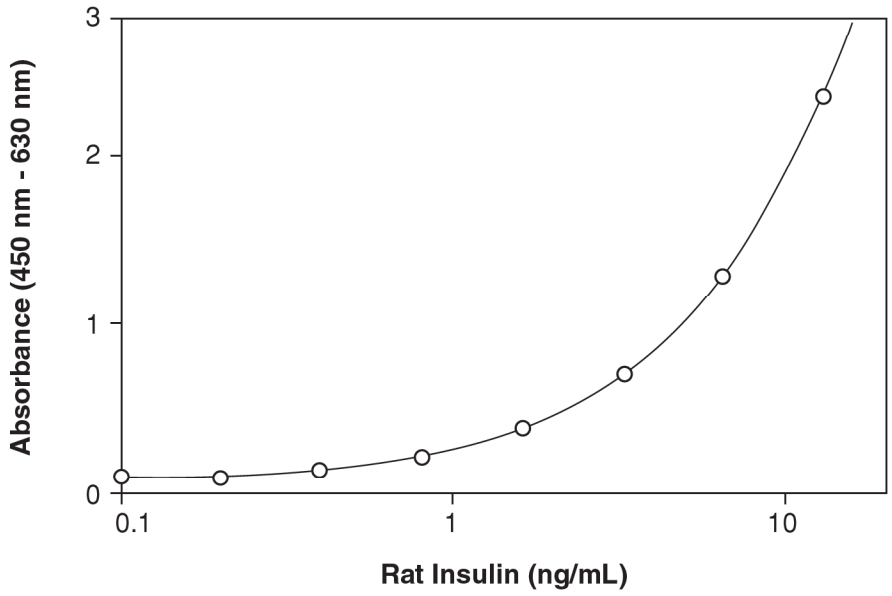
Third reaction:

8. Aspirate well contents and wash seven 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 and react for 40 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).

J.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 graph paper, construct the insulin standard curve by plotting the mean absorbance value for each standard on the Y axis versus the corresponding standard rat insulin concentration on the X axis. Figure 2 is an example of a typical standard curve generated by the wide range assay.
   ***Note**: A standard curve should be plotted every time the assay is performed.*
3. Rat insulin concentrations in the samples are interpolated using the standard curve and mean absorbance values for each sample.

Figure 2 A typical wide range standard curve (4-parameter fit)



K. High Range Assay (1 – 64 ng/mL)

***Note**: Bold underlined items denote the procedural differences between the low range and high range assay.*

K.1. Preparation of reagents

1. Prepare all the reagents for use according to Section I.1 under Low Range Assay except for the rat insulin stock solution and anti-insulin enzyme conjugate, which should be prepared as detailed below:
   Rat insulin stock solution - Reconstitute the "Rat Insulin Standard, Lyophilized" (marked "B") by careful addition of **40 µL** of distilled or deionized water to the vial. Invert the vial gently until the contents are completely dissolved. This stock solution contains 64 ng/mL of rat insulin. The reconstituted rat insulin stock solution is stable for one week at 2-8°C.
   Anti-insulin enzyme conjugate - For six modules (48 wells), prepare the needed volume of anti-insulin enzyme conjugate solution by mixing **2.0** mL of "Anti-Insulin Enzyme Conjugate Stock Solution" (marked "C"), **1.0** mL of "Enzyme Conjugate Diluent" (marked "D"), and **6.0 mL of "Sample Diluent" (marked "G")**. Mix completely to ensure a homogeneous and clear solution. Avoid foaming during mixing.
   ***Note**: The anti-insulin enzyme conjugate should be prepared just before the second reaction and must be used immediately.*

K.2. Preparation of working rat insulin standards

1. Pipette 40 µL of rat insulin stock solution (64 ng/mL) into a polypropylene microtube labeled 64 ng/mL.
2. Dispense 20 µL of sample diluent into six polypropylene microtubes labeled 1, 2, 4, 8, 16, and 32 ng/mL, respectively.
3. Dispense 20 µL of the 64 ng/mL standard into the 32 ng/mL microtube, and mix thoroughly.
4. Dispense 20 µL of the 32 ng/mL standard into the 16 ng/mL microtube, and mix thoroughly.
5. Repeat this dilution scheme using the remaining microtubes.
6. Dispense 20 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 5 Preparation of working rat insulin standards (high range assay)

	Rat Insulin concentration (ng/mL)							
	64	32	16	8	4	2	1	0
RISS*(µL)	40							
SD**(µL)	0	20	20	20	20	20	20	20
		20	20	20	20	20	20	
		↗	↗	↗	↗	↗	↗	
Total (µL)	40	40	40	40	40	40	40	20

RISS* : Rat Insulin Stock Solution (64 ng/mL)
SD** : Sample Diluent

K.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 microplates to the supporting frame.
2. In each well, dispense 95 µL of sample diluent (marked "G").
3. Pipette 5 µL samples (or 0, 1, 2, 4, 8, 16, 32, and 64 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 for 2 hours at 4°C.

Second reaction:

5. 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.

6. Dispense 100 µL per well of anti-insulin enzyme conjugate.
7. Cover the microplate with the plastic microplate cover and incubate for 30 minutes at room temperature.

Third reaction:

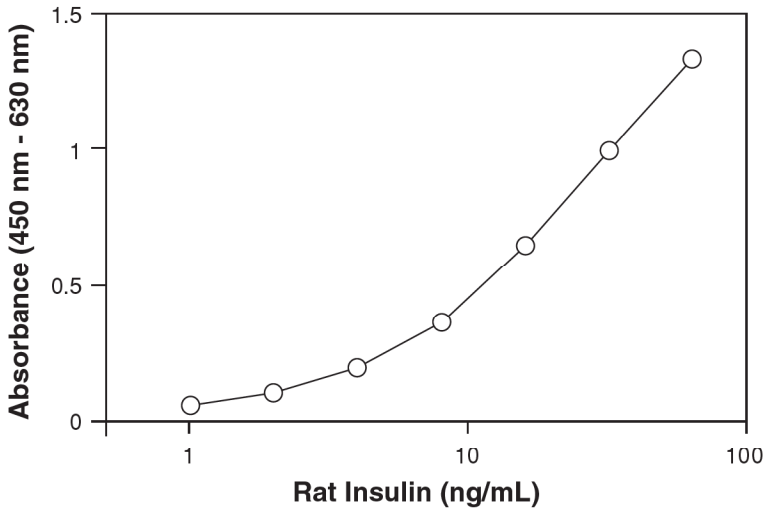
8. Aspirate well contents and wash seven times using 300 µL of wash buffer per well. After each wash, remove any remaining solution by inverting and tapping the plate firmly on a clean paper towel.
9. Immediately dispense 100 µL per well of enzyme substrate solution and react for **10 minutes** at room 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).

K.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 graph paper, construct the insulin standard curve by plotting the mean absorbance value for each standard on the Y axis versus the corresponding standard rat insulin concentration on the X axis. Figure 3 is an example of a typical standard curve generated by the high range assay.
   ***Note**: A standard curve should be plotted every time the assay is performed.*
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 (64 ng/mL or higher) should be diluted with the sample diluent and rerun.*

Figure 3 A typical high range standard curve (4-parameter fit)



L. Appendix

L.1.Performance characteristics (low range assay)

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 serum sample, the recovery was 100% ± 10%.
   When rat insulin was spiked in a 50 μL rat serum sample, the recovery was 100% ± 10%.
   When rat insulin was spiked in a 100 μL rat serum sample, the recovery was 100% ± 10%.

L.2. Increasing sensitivity (low range assay)

In cases in which samples are believed to contain an insulin concentration lower than 0.1 ng/mL (*i.e.* the lowest standard), the sample volume can be increased from 5 µL to a maximum of 100 µL to provide increased sensitivity. Using a 100 µL sample, the low range assay can detect a minimum insulin concentration of 5 pg/mL.

In such cases, the amount of sample diluent added in the first reaction (see 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 6, the more sample volume used, the lower the measurable insulin range.

TABLE 6 Increasing sensitivity through sample volume

Sample volume (µL)	Sample diluent (µL)	Total volume (µL)	Measurable insulin range (pg/mL)
5	95	100	100-6400
10	90	100	50-3200
20	80	100	25-1600
50	50	100	10- 640
100	0	100	5- 320

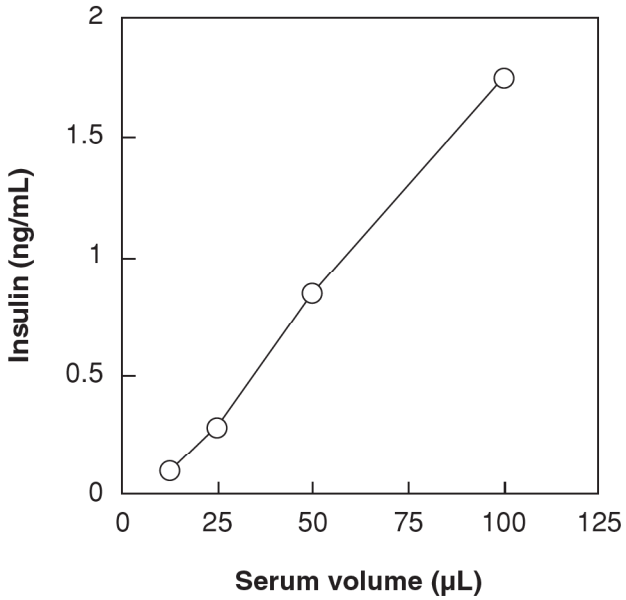
***Note**: Do not change the volume of rat insulin standard (5 µL) used, even if the sample volume is increased to greater than 5 µL.*

After adjusting the sample volume, run the rest of the low range assay as indicated in Section I.3. The insulin concentration of the sample should be calculated as follows:

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

An example of measuring a lower level insulin concentration (*i.e.* serum prepared from a Sterptozotocin (STZ)-treated mouse) is shown below in Figure 4.

Figure 4 Measurement of STZ-treated mouse serum



***Note**: Serum or plasma may be used with the Ultra Sensitive Rat Insulin ELISA kit. However, some samples may strongly inhibit the reaction between the anti-rat insulin antibody and the rat insulin. Therefore, when increasing sample volume, a preliminary test for insulin recovery should be performed by spiking the sample with a working rat insulin standard (see Section L.3. Rat insulin recovery test).*

L.3. Rat insulin recovery test

To determine the recovery of rat insulin, a minimum of three sample assays should be performed as illustrated in Table 7 (A, C, D or B, C, E).

TABLE 7 Example worksheet using a 5 µL sample

Unknown sample (µL)	Rat Insulin standard (µL)	Sample diluent (marked "G") (µL)	Total volume (µL)	Actual value* (ng/mL)
0	5 (0.4 ng/mL)	95	100	0.42 **A**
0	5 (0.8 ng/mL)	95	100	0.84 **B**
5	0 (0 ng/mL)	95	100	1.18 **C**
5	5 (0.4 ng/mL)	90	100	1.61 **D**
5	5 (0.8 ng/mL)	90	100	2.01 **E**

*Reflects the calculated sample concentration after adjusting the concentration read from the standard curve for any increase in sample volume (above 5 µL). See Section L.2. for further details.

Once the assays have been performed, use either of the calculations below to determine the insulin recovery:

Calculation 1

$$\text{Recovery (\%)} = \frac{\textbf{D}\ (1.61\ \text{ng/mL})}{\textbf{A}\ (0.42\ \text{ng/mL}) + \textbf{C}\ (1.18\ \text{ng/mL})} \times 100 = \textbf{100.6}$$

Calculation 2

$$\text{Recovery (\%)} = \frac{\textbf{E}\ (2.01\ \text{ng/mL})}{\textbf{B}\ (0.84\ \text{ng/mL}) + \textbf{C}\ (1.18\ \text{ng/mL})} \times 100 = \textbf{99.5}$$

L.4. Summary of reagent preparation

TABLE 8 Summary of reagent preparation

Reagent	Preparation Procedure	
	Low/Wide Range	High Range
A: Antibody-coated Microplate	Ready to use	
B: Rat Insulin Standard, Lyophilized	Dilute with 100 µL of water*	Dilute with 40 µL of water*
C: Anti-Insulin Enzyme Conjugate Stock Solution **D**: Enzyme Conjugate Diluent	For 6 modules** Reagent C - 3.6mL Reagent D - 1.8mL	For 6 modules** Reagent C - 2mL Reagent D - 1mL Reagent G - 6mL
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 1 L with water*	

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

* Distilled or deionized water.

** Prepare just before the second reaction.

L.5. Summary of Ultra Sensitive Rat Insulin ELISA kit assay

Affix the Antibody-coated Microplate (marked "A") to the frame.

↓

Dispense 95 µL of Sample Diluent (marked "G") per well.

↓

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

↓

Incubate the microplate for 2 hours at 4°C.

↓

Wash each well five times with wash buffer*.

↓

Dispense 100 µL of anti-insulin enzyme conjugate per well.

↓

Incubate the microplate for 30 min at room temperature.

↓

Wash each well seven times with wash buffer*.

↓

Dispense 100 µL of Enzyme Substrate Solution
(marked "E") per well.

↓

Incubate microplate at room temperature
while avoiding exposure to light.

↓

40 min - low/wide range assay 10 min - high range assay

↓ ↓

Stop the enzyme 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.

Warranty

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