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STANDARD OPERATING PROCEDURE PACKET

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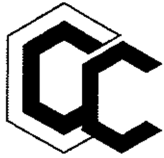

CRYSTAL CHEM INC.

Ultra Sensitive Mouse Insulin ELISA Kit Instructions

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

Catalog #90080

96 Assays

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

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A. *Intended Use*

The Ultra Sensitive Mouse Insulin ELISA kit is for the quantitative determination of insulin in mouse 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 mouse insulin. The following assays can be run using the Ultra Sensitive Mouse 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**
   Mouse 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/mouse 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 mouse insulin standard.

D. Kit Storage

1. Upon receipt of the Ultra Sensitive Mouse 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	Mouse 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 Mouse 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 for 20 min at 2,000 x *g*.

Serum: Collect blood, allow to clot, and centrifuge 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. Mouse insulin stock solution

   Reconstitute the "Mouse 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 mouse insulin. The reconstituted mouse 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 mouse insulin standards

1. Pipette 150 μL of sample diluent (marked "G") and 50 μL of mouse 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 mouse insulin standards (low range)

	Mouse insulin concentration (ng/mL)							
	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0
MISS*(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

MISS*: Mouse 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 mouse 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.*

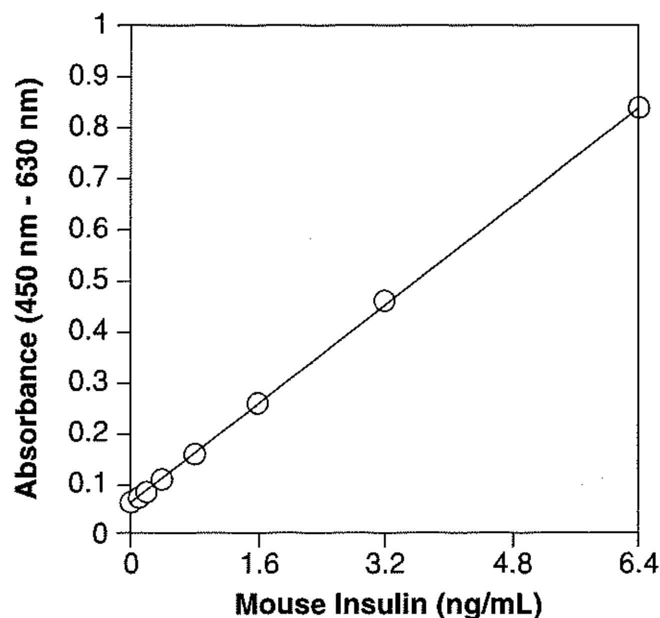
2. Using linear graph paper, construct the insulin standard curve by plotting the mean absorbance value for each standard on the Y axis versus the corresponding standard mouse insulin concentration on the X axis. Figure 1 is an example of a typical standard curve generated by the low range assay.

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

3. Mouse 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 mouse insulin standards

1. Pipette 50 µL of sample diluent (marked "G") and 50 µL of mouse 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 mouse insulin standards (wide range)

	Mouse Insulin concentration (ng/mL)								
	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0
MISS*(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

MISS*: Mouse 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 mouse 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.

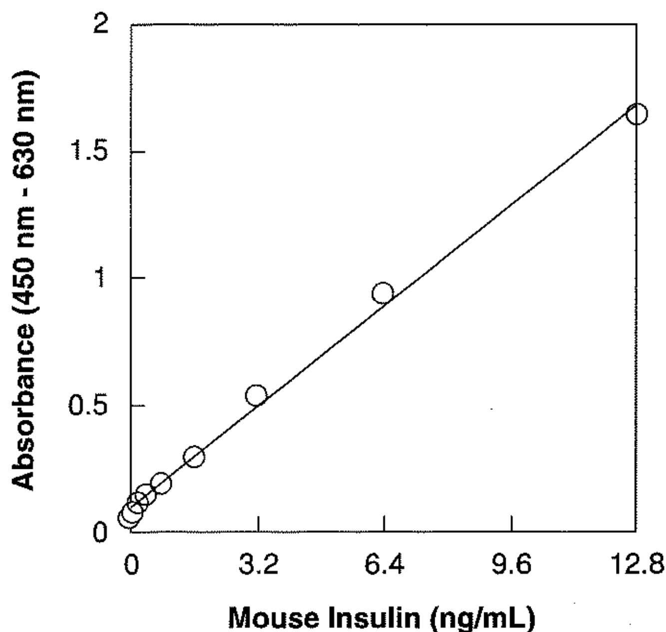
   Note: *If individual absorbance values differ from the mean by greater than 20%, performing the assay again is recommended. The mean absorbance of the 0 ng/mL standard should be less than 0.1.*

2. Using 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 mouse 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. Mouse 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 (linear 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 mouse insulin stock solution and anti-insulin enzyme conjugate, which should be prepared as detailed below:

   Mouse insulin stock solution - Reconstitute the "Mouse 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 mouse insulin. The reconstituted mouse 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 **3.0 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 mouse insulin standards

1. Pipette 40 µL of mouse 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 mouse insulin standards (high range)

	Mouse Insulin concentration (ng/mL)							
	64	32	16	8	4	2	1	0
MISS*(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

MISS*: Mouse 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 mouse 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:

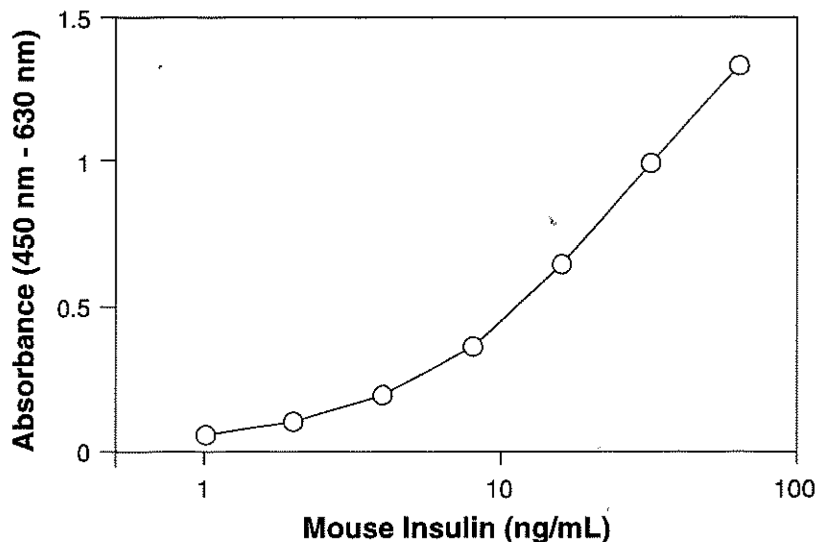
8. Aspirate well contents and wash seven times using 300 µL of wash buffer per well. After each wash, remove any remaining solution by inverting and tapping the plate firmly on a clean paper towel.
9. Immediately dispense 100 µL per well of enzyme substrate solution and react for **10 minutes** at room 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 mouse 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. Mouse 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 mouse insulin was spiked in a 5 μL mouse serum sample, the recovery was 100% ± 15%.
   When mouse insulin was spiked in a 50 μL mouse serum sample, the recovery was 100% ± 15%.
   When mouse insulin was spiked in a 100 μL mouse serum sample, the recovery was 100% ± 15%.

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 (ie., 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

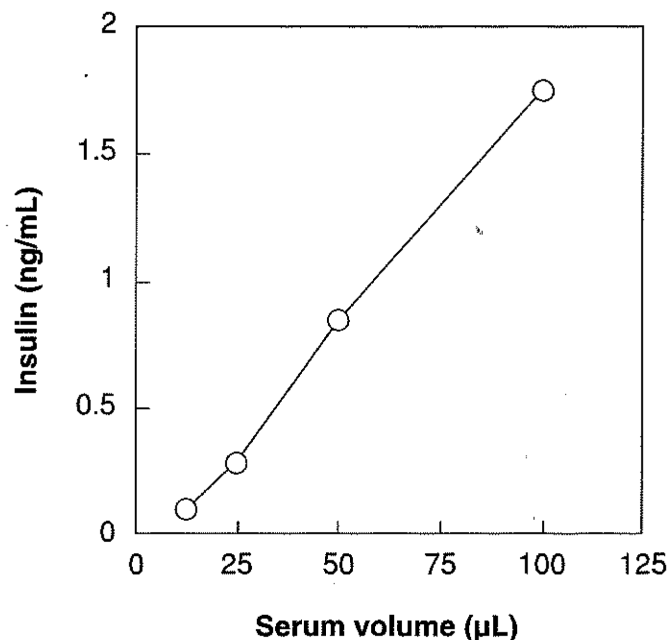
***Note**: Do not change the volume of mouse insulin standard (5 μL) used, even if the sample volume is increased to greater than 5 μL.*

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:

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

An example of measuring a lower level insulin concentration (ie. 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 Mouse Insulin ELISA kit. However, some samples may strongly inhibit the reaction between the anti-mouse insulin antibody and the mouse insulin. Therefore, when increasing sample volume, a preliminary test for insulin recovery should be performed by spiking the sample with a working mouse insulin standard (see Section L.3. - Mouse insulin recovery test).*

L.3. Mouse insulin recovery test

To determine the recovery of mouse 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)	Mouse Insulin standard (μL)	Sample diluent (marked "G") (μL)	Total volume (μL)	Actual value* (ng/mL)
0	5 (0.4 ng/mL)	95	100	0.36 **A**
0	5 (0.8 ng/mL)	95	100	0.79 **B**
5	0 (0 ng/mL)	95	100	0.48 **C**
5	5 (0.4 ng/mL)	90	100	0.77 **D**
5	5 (0.8 ng/mL)	90	100	1.13 **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

$$\text{Recovery (\%)} = \frac{\mathbf{D}\ (0.77\ \text{ng/mL})}{\mathbf{A}\ (0.36\ \text{ng/mL}) + \mathbf{C}\ (0.48\ \text{ng/mL})} \times 100 = \mathbf{91.7}$$

Calculation 2

$$\text{Recovery (\%)} = \frac{\mathbf{E}\ (1.13\ \text{ng/mL})}{\mathbf{B}\ (0.79\ \text{ng/mL}) + \mathbf{C}\ (0.48\ \text{ng/mL})} \times 100 = \mathbf{89.0}$$

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: Mouse 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 - 3mL
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: Washing 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 Mouse 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 mouse 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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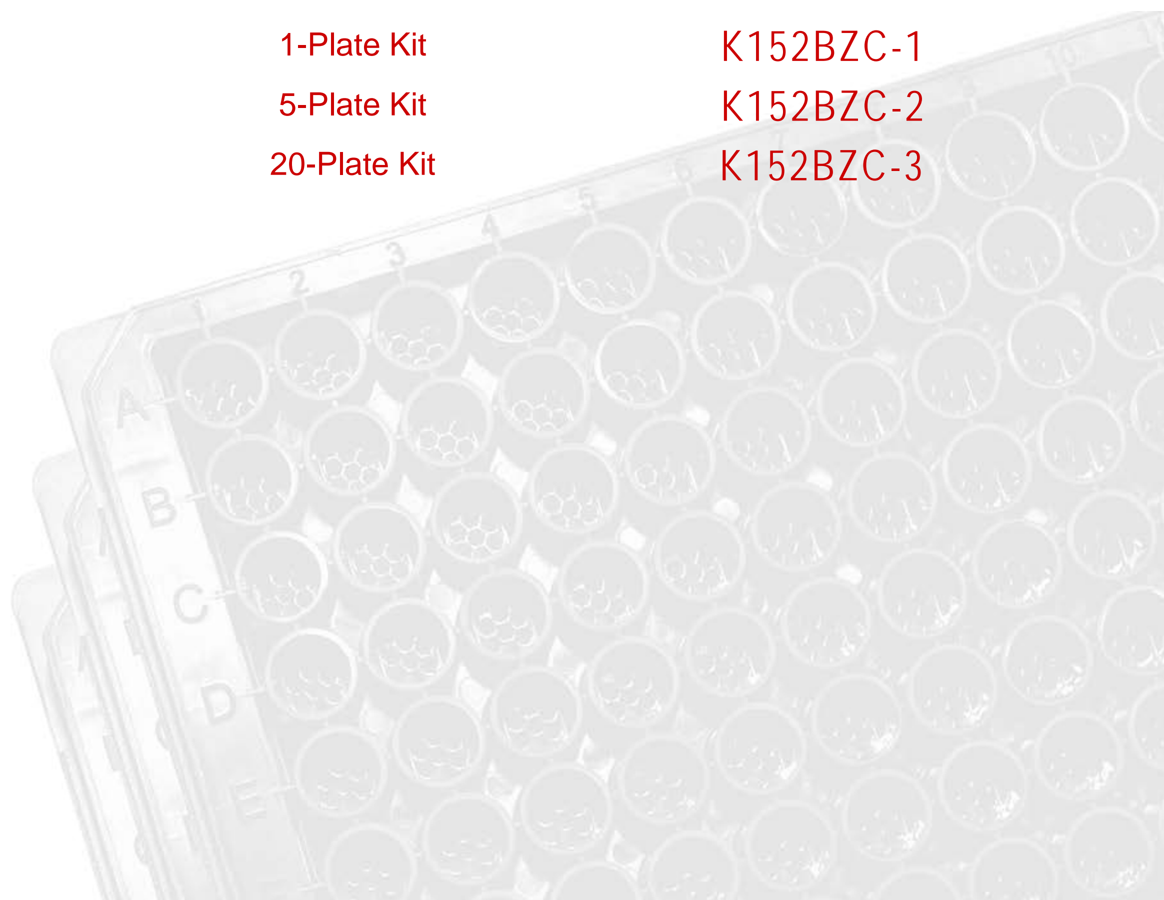
Meso Scale Discovery®

MULTI-ARRAY® Assay System

Mouse/Rat Insulin Kit



1-Plate Kit	K152BZC-1
5-Plate Kit	K152BZC-2
20-Plate Kit	K152BZC-3

Meso Scale Discovery Meso Scale Discovery Meso Scale Discovery Meso Scale Discovery Meso Scale Discovery Meso Scale Discovery Meso Scale Di

MSD Metabolic Assays

Mouse/Rat Insulin Kit

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

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Meso Scale Discovery
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Ordering Information

ordering information

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Introduction

introduction

Insulin is a 51-residue peptide hormone that is produced in the pancreas by β-cells of the islets of Langerhans. Insulin is involved in the regulation of carbohydrate, fat and protein metabolism. Lowered levels of insulin cause liver cells to convert glycogen back to glucose and secrete it into the blood. Insulin also has an effect on small vessel muscle tone, storage and release of (fat) triglycerides and cellular uptake of amino acids and electrolytes. Type 1 diabetes results when the β-cells are destroyed and no longer producing insulin resulting in high glucose levels in the blood. Patients with type 1 diabetes depend on exogenous insulin for their survival because of an absolute deficiency of the hormone; patients with type 2 diabetes have either relatively low insulin production or insulin resistance or both.

Principle of the Assay

principle of the assay

MSD® metabolic assays provide rapid and convenient methods for measuring the levels of protein targets within single small-volume samples. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. Our Mouse/Rat Insulin Assay detects insulin in a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with insulin antibody. The user adds the sample and a solution containing the labeled detection antibody—anti-insulin labeled with an electrochemiluminescent compound, MSD SULFO-TAG™ label—over the course of one or more incubation periods. Insulin in the sample binds to capture antibody immobilized on the working electrode surface; recruitment of the labeled detection antibody by bound analyte completes the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR instrument for analysis. Inside the SECTOR instrument, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to afford a quantitative measure of insulin present in the sample.

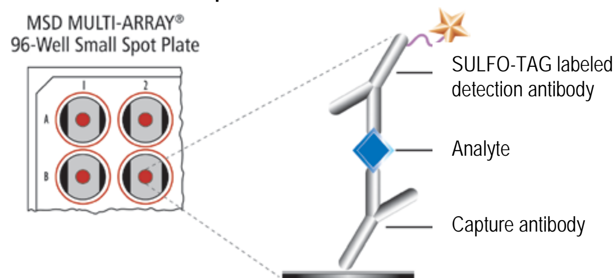


Figure 1. *Sandwich immunoassay on MSD platform*

Reagents Supplied

reagents supplied

Product Description	Storage	Quantity per Kit K152BZC-1	K152BZC-2	K152BZC-3
MULTI-ARRAY 96-well Mouse/Rat Insulin Plate(s) L452BZA-1	2-8°C	1 plate	5 plates	20 plates
SULFO-TAG Anti-m/r Insulin Antibody[1] (100X)	2-8°C	1 vial (60 µL)	1 vial (300 µL)	4 vials (300 µL ea)
Insulin Calibrator 5 µg/mL	≤-70°C	1 vial (15 µL)	5 vials (15 µL ea)	20 vials (15 µL ea)
Blocker A Kit R93AA-2 (250 mL)	RT	1 bottle (250 mL)	1 bottle (250 mL)	4 bottles (250 mL ea)
Diluent 100 R50AA-4 (50 mL) R50AA-2 (200 mL)	2-8°C	1 bottle (50 mL)	1 bottle (50 mL)	1 bottle (200 mL ea)
Diluent 17 R50KA-4 (6 mL) R50KA-3 (30 mL)	≤-10°C	1 bottle (6 mL)	1 bottle (30 mL)	4 bottles (30 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL) R92TC-2 (200 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	1 bottle (200 mL)

Required Materials and Equipment - not supplied

required materials and equipment — not supplied

- Deionized water for diluting concentrated buffers
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 25 to 150 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

Safety

safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines.

[1] Some SULFO-TAG labeled detection antibodies may be light-sensitive, so they should be stored in the dark.

VI Reagent Preparation

reagent preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

> **Important:** Upon first thaw, separate Diluent 17 into aliquots appropriate to the size of your assay needs. This diluent can go through up to three freeze-thaw cycles without significantly affecting the performance of the assay.

Prepare Blocker A Solution

Follow instructions included with the Blocker A Kit.

Prepare Calibrator and Control Solutions

Calibrator for the Mouse/Rat Insulin Assay is supplied at 5 µg/mL. For the assay, an 8-point standard curve is recommended with 3-fold serial dilution steps and a zero Calibrator. The table below shows the concentrations of the 8-point standard curve:

Standard	Insulin conc. (pg/mL)	Dilution Factor
Stock Cal. Vial	5000000	
STD-01	50000	100
STD-02	16667	3
STD-03	5556	3
STD-04	1852	3
STD-05	617	3
STD-06	206	3
STD-07	69	3
STD-08	0	n/a

To prepare this 8-point standard curve:

1) Prepare the highest Calibrator by transferring 10 µL of the Calibrator stock vial to 990 µL of Diluent 100.
2) Prepare the next Calibrator by transferring 100 µL of the diluted Calibrator to 200 µL of Diluent 100. Repeat 3-fold serial dilutions 5 additional times to generate 7 Calibrators.
3) The recommended 8th Standard is Diluent 100 (i.e. zero Calibrator).
4) Diluted Calibrators should be kept on ice prior to addition to the plate.

Note: The standard curve can be modified as necessary to meet specific assay requirements.

Preparation of Serum and Plasma Samples

The assay format requires 10 µL of sample per well. An adequate volume of each sample should be prepared depending upon desired number of replicates.

Prepare Detection Antibody Solution

The Detection Antibody is provided at 100X stock solution. The final concentration of the working Detection Antibody Solution should be at 1X. For each plate used, dilute a 50 µL aliquot of the stock Detection Antibody into 4.95 mL of Diluent 17.

Prepare Read Buffer

The Read Buffer should be diluted 4-fold in deionized water to make a final concentration of 1X Read Buffer T. Add 5 mL of 4X Read Buffer T to 15 mL of deionized water for each plate.

Prepare MSD Plate

This plate has been pre-coated with antibody for the analyte shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

VII Assay Protocol

assay protocol

1. **Addition of Blocker A Solution:** Dispense 150 µL of Blocker A Solution into each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.
2. **Wash and Addition of the Detection Antibody Solution followed by Sample or Calibrator:** Wash the plate 3 times with PBS-T. Dispense 40 µL of 1X Detection Antibody Solution into each well of the MSD plate. Immediately add 10 µL of sample or Calibrator into the appropriate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
3. **Wash and Read:** Wash the plate 3 times with PBS-T. Add 150 µL of 1X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates may be read immediately after the addition of Read Buffer.

Notes

Shaking a 96-well MSD MULTI-ARRAY plate typically accelerates capture at the working electrode.

Bubbles in the fluid will interfere with reliable reading of MULTI-ARRAY plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

VIII Analysis of Results

analysis of results

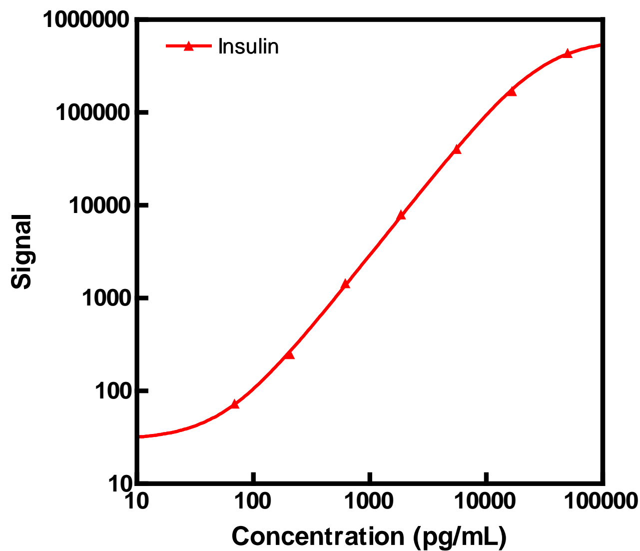
The Calibrators should be run in duplicate to generate a standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. The assays have a wide dynamic range (3–4 logs) which allows accurate quantification in many samples without the need for dilution. The MSD DISCOVERY WORKBENCH® analysis software utilizes a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting functionality is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve.

IX Typical Standard Curve

typical standard curve

The MSD Mouse/Rat Insulin Assay is designed for use with mouse and rat serum and plasma samples.

The following standard curve is an example of the dynamic range of the assay. The actual signals may vary. A standard curve should be run for each set of samples and on each plate for the best quantification of unknown samples.



	Insulin	
Conc. (pg/mL)	**Average Signal**	**%CV**
0	42	9.7
69	73	8.9
206	249	3.2
617	1441	4.3
1852	7968	8.9
5556	40493	11.3
16667	168989	4.2
50000	436781	1.6

X Sensitivity

sensitivity

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the zero Calibrator. The value below represents the average LLOD over multiple kit lots.

	Insulin
LLOD (pg/mL)	35

XI Spike Recovery

spike recovery

Serum, EDTA plasma, and heparin plasma were spiked with the Calibrators at multiple values throughout the range of the assay. Measured analyte represents average spike recovery in pooled mouse serum and plasma samples.
% Recovery = measured /expected x 100

	Spike Conc. (pg/mL)	% Recovery
Spiked Serum	500	89
	2500	85
	5000	81
Spiked EDTA Plasma	500	96
	2500	88
	5000	87
Spiked Heparin Plasma	500	95
	2500	92
	5000	88

XII Linearity

linearity

Linearity was measured by spiking Calibrator levels in pooled mouse plasma followed by subsequent dilution.
Percent recovery is calculated as the measured concentration divided by the concentration of the previous dilution (expected).
% Recovery = measured x dilution factor / expected x 100

	Fold Dilution	% Recovery
Serum	2	96
	4	107
	8	98
EDTA Plasma	2	103
	4	109
	8	103
Heparin Plasma	2	110
	4	110
	8	104

XIII Assay Components

assay components

Calibrator source: Recombinant human insulin
The insulin Calibrator has been anchored and referenced to international standards. The table below summarizes the reference information.

Analyte	WHO Standard Reference Number	WHO Standard Units / µg	MSD Calibrator 1µg = WHO Units	WHO Units
Insulin	66/304	0.023	0.023	IU

Capture Antibody	
Analyte	Mouse/Rat insulin
Source	Mouse monoclonal
Isoforms Recognized	Does not react with human proinsulin, rat or human C-peptide
Species cross-reactivity	Human, mouse, rat, porcine, bovine

Detection Antibody	
Analyte	Mouse/Rat insulin
Source	Mouse monoclonal
Isoforms Recognized	Does not react with human proinsulin, rat or human C-peptide
Species cross-reactivity	Human, mouse, rat, porcine, bovine

XIV References

references

1. Bristow AF, Das RE, Bangham DR. World Health Organization International Standards for highly purified human, porcine and bovine insulins. J Biol Stand. 1988 Jul;16(3):165-78.

2. Golla R, Seethala R. A sensitive, robust high-throughput electrochemiluminescence assay for rat insulin. J Biomol Screen. 2004 Feb;9(1):62-70

3. Plum L, Belgardt BF, Brüning JC. Central insulin action in energy and glucose homeostasis. J Clin Invest. 2006 Jul;116(7):1761-6

4. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature. 2001. Dec 13;414(6865):799-806

Summary Protocol

MSD 96-well MULTI-ARRAY Mouse/Rat Insulin Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the Mouse/Rat Insulin Assay.

Step 1 : Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

Prepare Blocker A Solution.

Prepare serum or plasma samples.

Prepare an 8-point standard curve using supplied Calibrator:

- The Calibrator should be diluted in Diluent 100.
- Dilute the stock Calibrator 1:100 in Diluent 100 then perform a series of 3-fold dilution steps and a no Calibrator blank.
- Diluted Calibrators should be kept on ice until use.

Note: *The standard curve can be modified as necessary to meet specific assay requirements.*

Prepare Detection Antibody Solution by diluting the 100X Anti-m/r Insulin Antibody to 1X in a final volume of 5.0 mL of Diluent 17 per plate.

Prepare 20 mL of 1X Read Buffer T by diluting 4X Read Buffer T with deionized water.

Step 2 : Add Blocker A Solution

Dispense 150 µL/well Blocker A Solution.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 1 hour.

Step 3 : Wash and Add Detection Antibody Solution Followed by Sample or Calibrator

Wash plate 3 times with PBS-T.

Dispense 40 µL/well 1X Detection Antibody Solution.

Immediately, Dispense 10 µL/well Calibrator or Sample.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

Step 4 : Wash and Read Plate

Wash plate 3 times with PBS-T.

Dispense 150 µL/well 1X Read Buffer T.

Analyze plate on SECTOR instrument.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
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H												