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# Plasma Insulin (LINCO ELISA) V.2

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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

**Summary:**

This Rat / Mouse Insulin ELISA kit is used for the non radioactive quantification of insulin in mouse and rat sera. Plasma samples may also be used but application to samples of other biological fluids may need validation by the user.

**Diabetic Complications:**

Cardiovascular

Retinopathy

Neuropathy

Nephropathy

Pediatric Endocrinology

Uropathy

Wound-Healing

## Materials

MATERIALS

RAT/MOUSE INSULIN ELISA KIT 96-Well Plate **LINCO ELISA kit Catalog #**EZRMI-13K

Plasma insulin is obtained from the LINCO ELISA kit.

**Manufacturer's Protocol below:**
RAT/MOUSE INSULIN ELISA KIT
96-Well Plate (Cat. # EZRMI-13K)

**Intended Use**
This Rat / Mouse Insulin ELISA kit is used for the non-radioactive quantification of insulin in mouse and rat sera. Plasma samples may also be used but application to samples of other biological fluids may need validation by the user. One kit is sufficient to measure 39 unknown samples in duplicate. This kit is for research purposes only.

**Principles of Procedure**
This assay is a Sandwich ELISA based, sequentially, on:
1) capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin,
2) wash away of unbound materials from samples,
3) binding of horseradish peroxidase to the immobilized biotinylated antibodies,
4) wash away of free enzyme conjugates, and
5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine.
The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

**Reagents Supplied**

Each kit is sufficient to run one 96-well plate including, in duplicates, background, 6 rat insulin standards, 2 quality controls and 39 unknown samples.

Microtiter Plate Coated with mouse monoclonal anti-rat insulin antibodies.
Quantity: 1 plate
Preparation: Ready to use
Adhesive Plate Sealer
Quantity: 1 Sheet
Preparation: Ready to use
10X Concentrate HRP Wash Buffer

10X concentrate of 50 mM Tris Buffered Saline containing Tween 20
Quantity: 50 ml/vial
Preparation: Dilute 10 times with de-ionized water
Standards
Rat Insulin in Assay Buffer: 0.2, 0.5 1, 2, 5, and 10 ng/ml.
Quantity: 0.1 ml/vial
Preparation: Ready to use
Quality Controls 1 and 2
Rat insulin in QC buffer.
Quantity: 0.1 ml/vial
Preparation: Ready to use
Matrix Solution
Charcoal stripped pooled mouse serum
Quantity: 0.5 ml
Preparation: Ready to use
Assay Buffer
0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium Azide, and 1% BSA
Quantity: 20 ml
Preparation: Ready to use
Detection Antibody
Pre-titered biotinylated anti-insulin antibody
Quantity: 10 ml
Preparation: Ready to use
Enzyme Solution
Pre-titered streptavidin-horseradish peroxidase conjugate in buffer
Quantity: 12 ml/vial
Preparation: Ready to use
Substrate (Light sensitive, avoid unnecessary exposure to light) 3, 3',5,5'-tetramethylbenzidine in Buffer
Quantity: 12 ml
Preparation: Ready to use.
Stop Solution
0.3 M HCl
Quantity: 12 ml
Preparation: Ready to Use
Storage and Stability

Prior to use, all components in the kit can be stored up to 2 weeks at 2-8°. For longer storage (>2 weeks), freeze diluted TBS wash buffer, insulin standards, quality controls, and matrix solution at -20ºC. Minimize repeated freeze and thaw of the insulin standards, quality controls and matrix solution. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

**Reagent Precautions**

***Sodium Azide***

Sodium azide has been added to certain reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

***Hydrochloric Acid***

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

**Materials Required but not provided**

Pipettes with tips: 10ml - 100 ml
Multi-Channel Pipettes: 50 ~ 300 ml
Reagent Reservoirs
Vortex Mixer
Refrigerator
Deionized Water
Microtiter Plate Reader capable of reading absorbency at 450 nm and 590 nm
Orbital Microtiter Plate Shaker
Absorbent Paper or Cloth
Sample Collection and Storage

To prepare serum, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4º±2ºC. Transfer and store serum samples in separate tubes. Date and identify each sample.
Use freshly prepared serum or aliquot and store samples at -20º± 5ºC for later use. For long-term storage, keep at -70ºC. Avoid freeze/thaw cycles. To prepare plasma sample, whole blood should be collected into centrifuge tubes containing enough K3EDTA to achieve a final concentration of 1.735 mg/ml and centrifuged immediately after collection. Observe same precautions in the preparation of serum samples. If heparin is to be used as anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined. Avoid using samples with gross hemolysis or lipemia.

## Troubleshooting

## Assay Procedure

1 Pre-warm all reagents to room temperature immediately before setting up the assay.

Dilute the 10x concentrated TBS wash buffer 10 fold by mixing the entire content of buffer with 450ml de-ionized water. Remove the microtiter assay plate from the foil pouch and wash each well 3 times with 300 ml of diluted TBS wash buffer per wash. Decant wash buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step. If automated machine is used for assay, follow the manufacturer's instructions for all washing steps described in this protocol.

Transfer detection antibody solution to a reagent reservoir and add 80 ml of this solution to each well with a multi-channel pipette. If samples to be assayed are serum or plasma, add 10 ml matrix solution to the NSB, Standard, and Control wells (Option A). If samples are free of significant serum matrix components, add 10 ml assay buffer instead (Option B). Refer to the Microtiter Plate Arrangement section for suggested well orientations.

• Add 10 ml assay buffer to each of the sample wells.

• Add 10 ml assay buffer to the NSB wells. Add in duplicate 10 ml rat insulin standards in the order of ascending concentration to the appropriate wells.

• Add 10 ml QC1 and 10 ml QC2 to the appropriate wells.

• Add sequentially 10 ml samples of the unknown samples in duplicates to the remaining wells. For best result all additions should be completed within one hour. Cover the plate with plate sealer and incubate at room temperature for 2 hours on a orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

• Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.

• Wash wells 3 times with diluted TBS wash buffer, 300 ml per well per wash.

• Decant and tap after each wash to remove residual buffer.

• Add 100 ml enzyme solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the microtiter plate shaker.

• Remove sealer, decant solutions from the plate nd tap plate to remove the residual fluid.

• Wash wells 6 times with diluted TBS buffer, 300 ml per well per wash. Decantand tap after each wash to remove residual buffer.

• Add 100 ml of substrate solution to each well, cover plate with sealer and shake in the plate shaker for 15 minutes. Blue color should be formed in wells of insulin standards with intensity proportional to increasing concentrations of insulin.

• Remove sealer and add 100 ml stop solution ***[CAUTION: CORROSIVE SOLUTION]*** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well. Record the difference of absorbance units. Alternatively, the increase in blue color can be monitored at 630 nm wavelength and the stop solution to be added when the absorbance of the highest standard wells reached 0.8 to 0.9. Assay Procedure for Rat / Mouse Insulin ELISA Kit (Cat. # EZRMI-13K)

Option A: For Samples with significant Serum Matrix Effect

Option B: For Samples without significant Serum Matrix Effect

## Microtiter Plate Arrangement

2 **Calculations**

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function. Graph the reference curve for sample interpretation by plotting the absorbance unit of 450nm, less that of 590nm, on the Y-axis against the concentration of rat insulin standards on the X-axis.

**[Note: When sample volumes assayed differ from 10 ml, an appropriate mathematical adjustment must be made to accommodate for the dilution factor ( e.g., if 5 ml of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 10 ml, compensate the volume deficit with either matrix solution or assay buffer, whichever is appropriate.]**

**Interpretation**

The assay will be considered accepted when all Quality Control values fall within the calculated Quality Control Range. If any QC's fall outside the control range, review results with a supervisor. If the difference between duplicate results of a sample is >15% CV, repeat the sample. The limit of sensitivity of this assay is 0.2 ng/ml (35 pM) insulin (10 ml sample size). The appropriate range of this assay is 0.2 ng/ml to 10 ng/ml insulin (10 ml sample size). Any result greater than 10 ng/ml in a 10 ml sample assayed should be repeated on dilution using either matrix solution or assay buffer, whichever is appropriate, as diluent until it falls within range.

**Assay Characteristics**

***Sensitivity***

The lowest level of Insulin that can be detected by this assay is 0.2 ng/ml (35 pM) insulin when using a 10 ml sample size.

***Specificity***

The specificity (also known as selectivity) of the analytical test is its ability to selectively measure the analytes in the presence of other like components in the sample matrix.

Rat Insulin 100% [ED(50) = 1.57 nM]
Porcine Insulin 102%
Bovine insulin 78%
Ovine Insulin 106%
Human Insulin 106%
Human Proinsulin 52%
Des(64,65) Human Proinsulin 101%
Des(31,32) Human Proinsulin 69%
Porcine Proinsulin 57%
Bovine Proinsulin 56%
Human IGF-I n.d.*
Human IGF-II n.d.*
Porcine Glucagon n.d.*
Human C-Peptide n.d.**
Rat C-Peptide n.d.*
Rat Leptin n.d.*
Mouse Leptin n.d.*

n.d.: Not detectable at concentrations up to * - nM.

***Precision***

Sample
Number Mean Insulin Level (ng/ml) Assay Variation (% CV) Intra-assay Inter-assay

| | | | |
|---|---|---|---|
| Mouse serum #1 | 0.32 | 8.35 | 17.9 |
| Mouse serum #2 | 1.69 | 0.92 | 6.03 |
| Mouse serum #3 | 3.45 | 1.92 | 7.64 |
| Rat serum #1 | 1.15 | 3.22 | 6.95 |
| Rat serum #2 | 2.32 | 1.33 | 6.71 |
| Rat serum #3 | 3.65 | 1.17 | 9.23 |

The assay variations of Linco Mouse/Rat Insulin ELISA kit were studied on three mouse and three rat serum samples with varying concentrations of endogenous analyte. The intra-assay variations are calculated from six duplicate determinations in an assay. The inter-assay variations are calculated from results of 5 separate assays with duplicate samples in each assay.

**Dilutional Linearity**

| Serum Sample # | Dilution Factor | Insulin Level (ng/ml) | Observed Expected (ng/ml) | % of Expected |
|---|---|---|---|---|
| Mouse Serum #1 | -- | 2.06 | 2.06 | 100 |
| 2x | 1.84 | | | 89 |
| 4x | 2.20 | | | 107 |
| 8x | 3.12 | | | 152 |
| Mouse Serum #2 | -- | 2.98 | 2.98 | 100 |
| 2x | 2.84 | | | 95 |
| 4x | 3.08 | | | 103 |
| 8x | 3.76 | | | 126 |
| Mouse Serum #3 | -- | 2.95 | 2.95 | 100 |
| 2x | 2.94 | | | 100 |
| 4x | 3.08 | | | 96 |
| 8x | 3.92 | | | 105 |
| Rat Serum #1 | -- | 3.78 | 3.78 | 100 |
| 2x | 3.16 | | | 84 |
| 5x | 3.00 | | | 79 |
| 10x | 3.40 | | | 90 |
| Rat Serum #2 | -- | 3.78 | 3.78 | 100 |
| 2x | 3.16 | | | 84 |
| 5x | 3.00 | | | 79 |
| 10x | 3.40 | | | 90 |
| Rat Serum #3 | -- | 3.42 | 3.42 | 100 |
| 2x | 3.12 | | | 91 |
| 5x | 3.15 | | | 92 |
| 10x | 3.90 | | | 114 |

Three mouse and three rat serum samples are diluted each with matrix solution to various degrees as indicated and assayed for insulin levels along with neat samples of each serum. Measured insulin levels are corrected for dilution factors and reported as observed insulin level.

**Recovery**

Spike & Recovery of Insulin in Serum Samples.

| Serum Sample # | Serum Sample # | Rat Insulin | Recovery (%) of Spiked Insulin |
|---|---|---|---|
| | Added (ng/ml) | Observed (ng/ml) | |
| Mouse Serum #1 | 0 | 0.33 | -- |
| | 0.5 | 0.83 | 100 |
| | 2 | 2.15 | 91 |
| | 5 | 5.07 | 95 |
| Mouse Serum #2 | 0 | 1.78 | -- |
| | 0.5 | 2.20 | 84 |
| | 2 | 3.43 | 83 |
| | 5 | 6.16 | 88 |
| Mouse Serum #3 | 0 | 1.01 | -- |
| | 0.5 | 1.49 | 96 |
| | 2 | 2.91 | 95 |
| | 5 | 5.95 | 99 |
| Rat Serum #1 | 0 | 1.06 | -- |
| | 0.5 | 1.57 | 102 |
| | 2 | 2.86 | 90 |
| | 5 | 5.88 | 96 |
| Rat Serum #2 | 0 | 10.7 | -- |
| | 0.5 | 1.53 | 92 |
| | 2 | 2.95 | 94 |
| | 5 | 6.01 | 99 |
| Rat Serum #3 | 0 | 0.99 | -- |
| | 0.5 | 1.45 | 92 |
| | 2 | 2.69 | 85 |
| | 5 | 5.40 | 88 |

Rat insulin at indicated levels was added to three mouse and three rat serum samples and the resulting insulin content of each sample was assayed by ELISA. The % of recovery = [(observed insulin level after spike – observed insulin level before spike) / spiked level of insulin] x 100%. Mean±S.D. of recovery rate at spiked insulin level of 0.5, 2, and 5 ng/ml is 93 ± 8%, 90 ± 6% and 94 ± 6% in mouse serum and 95 ± 6%, 90 ± 5% and 94 ± 7% in rat serum, respectively.

## Troubleshooting Guide

3 To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practices. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started, all steps should be completed with precise timing and without interruption. Avoid cross contamination of any reagents or samples to be used in the assay.
Make sure that all reagents and samples are added to the bottom of each well. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or well cross contamination due to inappropriate mixing. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings. High absorbance in background or blank wells could be due to 1.) well cross contamination by standard solution or sample and 2.) inadequate washing of wells with TBS.

***Replacement Reagents***

Reagents Cat. #
Microtiter Plate EP13
10X Wash Buffer Concentrate (50 ml) EWB-HRP
Rat Insulin Standards E8013-K
Quality Controls 1 & 2 E6013
Matrix Solution EMTX-RMI
Assay Buffer AB-PHK
Detection Antibody E1013
Enzyme Solution EHRP-3
Substrate ESS-TMB2
Stop Solution ET-TMB