User Guide

Rat/Mouse Insulin ELISA Kit

96-Well Plate

EZRMI-13K

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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 Use Only. Not for use in Diagnostic Procedures.

Principles of Assay

This assay is a Sandwich ELISA based, sequentially, on:

- 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
- Wash away of unbound materials from samples
- Binding of horseradish peroxidase to the immobilized biotinylated antibodies
- Wash away of free enzyme conjugates
- 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 590 nm, 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 and contains the following reagents:

Note: Store all reagents at 2-8 °C

Reagents Supplied	Volume	Quantity	Cat. No.
Rat/Mouse Insulin ELISA Plate with 2 plate sealers	-	1 plate 2 sealers	EP13
Rat/Mouse Insulin Standards 0.2, 0.5, 1, 2, 5 and 10 ng/mL	0.25 mL/vial	6 vials	E8013-K
Quality Controls 1 and 2	0.25 mL/vial	1 vial each	E6013-K
Matrix Solution	0.5 mL	1 vial	EMTX-RMI
Assay Buffer	20 mL	1 vial	AB-PHK
10X HRP Wash Buffer Concentrate.	50 mL	2 bottles	EWB-HRP
Rat/Mouse Insulin Detection Antibody	10 mL	1 vial	E1013
Enzyme Solution	12 mL	1 vial	EHRP-88
Substrate Solution **Note:** Minimize light exposure.	12 mL	1 vial	ESS-TMB2
Stop Solution **Caution:** Corrosive Solution	12 mL	1 vial	ET-TMB

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Standards and controls can be frozen for future use but repeated freeze/thaw cycles should be avoided. 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 some reagents as a preservative. Although the concentrations are low, Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Hydrochloric Acid

Hydrochloric acid is corrosive and can cause eye and skin burns. Harmful if swallowed. Causes respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

Note: See Full Labels of Hazardous components on next page.

Symbol Definitions

Ingredient	Cat. No.	Full Label	
Rat/Mouse Insulin ELISA Plate	EP13		**Warning:** Causes skin irritation. May cause an allergic skin reaction. Causes serious eye irritation. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention.
Stop Solution	ET-TMB		**Warning:** May be corrosive to metals.
10X HRP Wash Buffer Concentrate	EWB-HRP		**Warning:** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

Materials Required (Not Provided)

- Multi-channel Pipettes and pipette tips: 50 µL-300 µL
- Pipettes and pipette tips: 10 µL-100 µL
- Reagent Reservoirs
- Vortex Mixer
- Refrigerator
- De-ionized 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

1. 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.
2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x *g* for 15 minutes at 4 ± 2°C.
3. Transfer and store serum samples in separate tubes. Date and identify each sample.
4. Use freshly prepared serum or aliquot and store samples at −20 ± 5 °C for later use. For long-term storage, keep at −70 °C. Avoid freeze/thaw cycles.
5. To prepare plasma sample, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe same precautions in the preparation of serum samples.
6. 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.
7. Avoid using samples with gross hemolysis or lipemia.

Rat/Mouse Insulin ELISA Assay Procedure

Warm all reagents to room temperature before setting up the assay.

1. Dilute 10X concentrated HRP wash buffer 10-fold by mixing the entire contents of both buffer bottles with 450 mL de-ionized (dilute both bottles with 900 mL deionized water).
2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8 °C. Assemble the strips in an empty plate holder. Add 300 µL diluted Wash Buffer to each well of the plate. Decant Wash Buffer and remove the residual volume by inverting the plate and tapping it smartly onto absorbent towels several times. Repeat wash procedure 2 additional times. **Do not let wells dry before proceeding to the next step.** If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.
3. Add 10 µL of Assay Buffer to each of the Blank and sample wells (refer to Microtiter Plate Arrangement).
4. If samples to be assayed are serum or plasma, add 10 µL Matrix Solution to the Blank, Standard and Control wells (Option A). If samples are free of significant serum matrix components, add 10 µL Assay Buffer instead (Option B).
5. Add in duplicate 10 µL Rat Insulin Standards in order or ascending concentrations to the appropriate wells.
6. Add in duplicate 10 µL QC1 and 10 µL QC2 to the appropriate wells.
7. Add sequentially 10 µL of the unknown samples in duplicate to the remaining wells.

8. Add 80 µL Detection Antibody to all 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 an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

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

10. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

11. Add 100 µL 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.

12. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.

13. Wash well 6 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

14. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake in the plate shaker for approximately 5 to 20 minutes. Blue color should be formed in wells of Insulin Standards with intensity proportional to increasing concentrations of insulin.

    Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time. One can measure the color development using 370 nm filter, if available on the spectrophotometer. When absorbance is between 1.2 and 1.8 at 370 nm, the stop solution can be added to terminate color development.

15. Remove sealer and add 100 µL 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. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. 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.

Assay Procedure for Rat/Mouse Insulin ELISA Kit

Option A: For Samples with Significant Serum Matrix Effect

Well #	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 8	Step 8-10	Step 11	Step 11-13	Step 14		Step 15	
			Assay Buffer	Matrix Solution	Standards/ QCs/Samples	Detection Ab		Enzyme Solution		Substrate		Stop Solution	
A1, B1	Dilute both bottles of 10X Wash Buffer with 900 mL Deionized Water.	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	10 µL	10 µL	-	80 µL	Seal, Agitate, Incubate 2 hours at Room Temperature on a plate shaker. Wash 3X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 30 minutes at Room Temperature on a plate shaker. Wash 6X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 5-20 mins at Room Temperature on a plate shaker.	100 µL	Read Absorbance at 450 nm and 590 nm.
C1, D1			-	10 µL	10 µL of 0.2 ng/mL Standard								
E1, F1			-	10 µL	10 µL of 0.5 ng/mL Standard								
G1, H1			-	10 µL	10 µL of 1 ng/mL Standard								
A2, B2			-	10 µL	10 µL of 2 ng/mL Standard								
C2, D2			-	10 µL	10 µL of 5 ng/mL Standard								
E2, F2			-	10 µL	10 µL of 10 ng/mL Standard								
G2, H2			-	10 µL	10 µL of QC 1								
A3, B3			-	10 µL	10 µL of QC 2								
C3, D3			10 µL	-	10 µL of Sample								

Option B: For Samples without Significant Serum Matrix Effect

Well #	Step 1	Step 2	Step 3-4	Step 5-7	Step 8	Step 8-10	Step 11	Step 11-13	Step 14		Step 15	
			Assay Buffer	Standards/ QCs/Samples	Detection Ab		Enzyme Solution		Substrate		Stop Solution	
A1, B1	Dilute both bottles of 10X Wash Buffer with 900 mL Deionized Water.	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	20 µL	-	80 µL	Seal, Agitate, Incubate 2 hours at Room Temperature on a plate shaker. Wash 3X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 30 minutes at Room Temperature on a plate shaker. Wash 6X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 5-20 mins at Room Temperature on a plate shaker.	100 µL	Read Absorbance at 450 nm and 590 nm.
C1, D1			10 µL	10 µL of 0.2 ng/mL Standard								
E1, F1			10 µL	10 µL of 0.5 ng/mL Standard								
G1, H1			10 µL	10 µL of 1 ng/mL Standard								
A2, B2			10 µL	10 µL of 2 ng/mL Standard								
C2, D2			10 µL	10 µL of 5 ng/mL Standard								
E2, F2			10 µL	10 µL of 10 ng/mL Standard								
G2, H2			10 µL	10 µL of QC 1								
A3, B3			10 µL	10 µL of QC 2								
C3, D3			10 µL	10 µL of Sample								

Microtiter Plate Arrangement

Rat/Mouse Insulin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	2 ng/mL	QC 2									
B	Blank	2 ng/mL	QC 2									
C	0.2 ng/mL	5 ng/mL	Sample									
D	0.2 ng/mL	5 ng/mL	Sample									
E	0.5 ng/mL	10 ng/mL	Sample									
F	0.5 ng/mL	10 ng/mL	Sample									
G	1 ng/mL	QC1	Sample									
H	1 ng/mL	QC1	Etc.									

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 450 nm, less that of 590 nm, on the Y-axis against the concentration of rat insulin standards on the X-axis.

Note: When sample volumes assayed differ from 10 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (for example, if 5 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 10 µL, 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 0.1 ng/mL (17.5 pM) insulin (10 µL sample size).
- The appropriate range of this assay is 0.1 ng/mL to 10 ng/mL insulin (10 µL sample size). Any result greater than 10 ng/mL in a 10 µL 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 0.1 ng/mL (17.5 pM) insulin when using a 10 µL 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

Precision

Sample Number	Mean Insulin Level (ng/mL)	Mean 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 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		
		Observed (ng/mL	Expected (ng/mL)	% of Expected
Mouse Serum #1	-	2.06		100
	2x	1.84		89
	4x	2.20		107
	8x	3.12	2.06	152
Mouse Serum #2	-	2.98		100
	2x	2.84		95
	4x	3.08		103
	8x	3.76	2.98	126
Mouse Serum #3	-	2.95		100
	2x	2.94		100
	4x	3.08		104
	8x	3.92	2.95	133
Rat Serum #1	-	4.22		100
	2x	3.80		90
	5x	3.55		84
	10x	4.70	4.22	111
Rat Serum #2	-	3.78		100
	2x	3.16		84
	5x	3.00		79
	10x	3.40	3.78	90
Rat Serum #3	-	3.42		100
	2x	3.12		91
	5x	3.15		92
	10x	3.90	3.42	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.

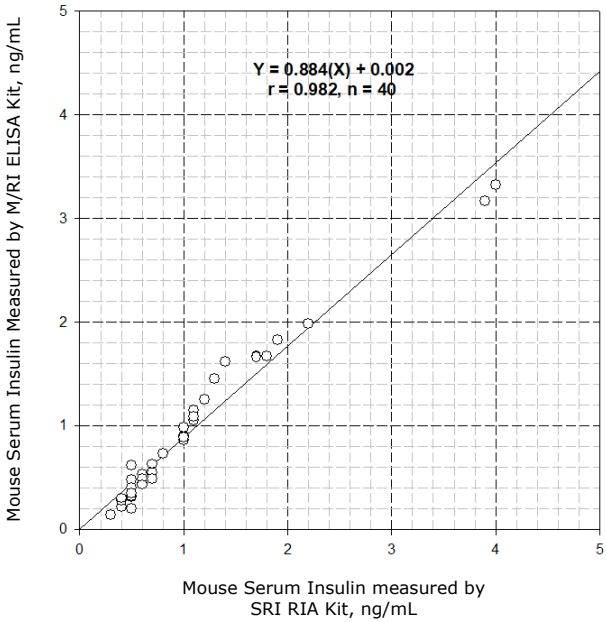
Spike Recovery of Insulin in Serum Samples

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	1.07	-
	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

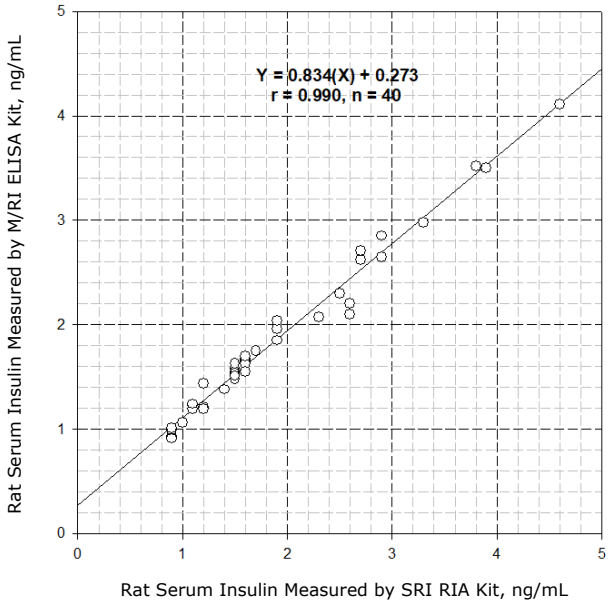
Rat insulin at indicated levels was added to three mouse and three rat serum samples and the resulting insulin content of each sample was assayed by ELISA. The % of recovery = [(observed insulin level after spike - observed insulin level before spike) / spiked level of insulin] x 100%. Mean ± S.D. of 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.

Correlation Graph

Mouse Serum Insulin Assay: Correlation of Results by RIA and ELISA Methods



Rat Serum Insulin Assay: Correlation of Results by RIA and ELISA Methods



Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

- 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 practice.
- 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 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 cross well contamination due to inappropriate mixing.
- Remove any air bubbles 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
    - cross well contamination by standard solution or sample, or
    - inadequate washing of wells with Wash Buffer

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
Rat/Mouse Insulin ELISA Plate	EP13
10X HRP Wash Buffer Concentrate	EWB-HRP
Rat/Mouse Insulin Standards	E8013-K
Rat/Mouse Insulin Quality Controls 1 and 2	E6013-K
Matrix Solution	EMTX-RMI
Assay Buffer	AB-PHK
Rat/Mouse Insulin Detection Antibody	E1013
Enzyme Solution	EHRP-88
Substrate	ESS-TMB2
Stop Solution	ET-TMB

Notice

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