Rat / Mouse Insulin

96 Well Plate Assay

Cat. # EZRMI-13K

RAT / MOUSE INSULIN ELISA KIT

96-Well Plate (Cat. # EZRMI-13K)

Millipore • 6 Research Park Drive • St. Charles, Missouri 63304 USA
Phone: 636-441-8400 • Toll Free U.S.: 800-MILLIPORE • Fax: 636-441-8050
Techserve_dd@millipore.com • www.millipore.com

Rat / Mouse Insulin ELISA Kit
96-Well Plate (Cat. # EZRMI-13K)

I. 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 purpose only.***

II. 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.

III. 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.

A. Rat/Mouse Insulin ELISA Plate

Coated with mouse monoclonal anti-rat insulin antibodies.
Quantity: 1 plate
Preparation: Ready to use.
Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C.

B. Adhesive Plate Sealer

Quantity: 1 sheet
Preparation: Ready to use.

C. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
Quantity: Two bottles containing 50 mL each
Preparation: Dilute 10 times with de-ionized water.

III. REAGENTS SUPPLIED (continued)

D. Rat/Mouse Insulin Standards

Rat insulin in Assay Buffer: 0.2, 0.5, 1, 2, 5 and 10 ng/mL.
Quantity: 0.25 mL/vial
Preparation: Ready to use.

E. Rat/Mouse Insulin Quality Controls 1 and 2

Rat insulin in QC buffer.
Quantity: 0.25 mL/vial
Preparation: Ready to use.

F. Matrix Solution

Charcoal stripped pooled mouse serum
Quantity: 0.5 mL
Preparation: Ready to use.

G. 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.

H. Rat/Mouse Insulin Detection Antibody

Pre-titered biotinylated anti-insulin antibody.
Quantity: 10 mL
Preparation: Ready to use.

I. Enzyme Solution

Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.
Quantity: 12 mL
Preparation: Ready to use.

J. Substrate (Light sensitive, avoid unnecessary exposure to light)

3, 3', 5, 5'-tetramethylbenzidine in buffer.
Quantity: 12 mL
Preparation: Ready to use.

K. Stop Solution

0.3 M HCl
Quantity: 12 mL
Preparation: Ready to use.

IV. STORAGE AND STABILITY

Prior to use, all components in the kit can be stored up to 2 weeks at 2 - 8°C. For longer storage (> 2 weeks), freeze diluted Wash Buffer, Insulin Standards, Quality Controls, and Matrix Solution at ≤-20°C. Minimize repeated freeze and thaw of the Insulin Standards, Quality Controls and Matrix Solution. Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

A. Sodium Azide

Sodium Azide has been added to certain reagents as a preservative. Although the concentrations are low, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. Flush with a large volume of water to prevent azide build-up.

B. 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 eye. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipette with tips, 10µL - 100 µL.
2. Multi-channel Pipette: 50 µL ~ 300 µL
3. Reagent Reservoirs
4. Vortex Mixer
5. Refrigerator
6. De-ionized Water
7. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590nm
8. Orbital Microtiter Plate Shaker
9. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

A. 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.

B. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2°C.

C. Transfer and store serum samples in separate tubes. Date and identify each sample.

D. 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.

VII. SAMPLE COLLECTION AND STORAGE (continued)

E. 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.

F. 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.

G. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

Pre-warm all reagents to room temperature prior to setting up assay.

1. Dilute the 10X Wash Buffer concentrate 10 fold by mixing the entire content of each bottle of Wash Buffer with 450mL de-ionized water. (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 strips in an empty plate holder and wash each well 3 times with 300 µL of diluted 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.

3. Add 10 µL Assay Buffer to the NSB wells and to each of the sample wells. Refer to Section IX for suggested well orientations.

4. If samples to be assayed are serum or plasma, add 10 µL Matrix Solution to the NSB, 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 the order of ascending concentration to the appropriate wells.

6. Add 10 µL QC1 and 10 µL QC2 to the appropriate wells.

7. Add sequentially 10 µL samples of the unknown samples in duplicates 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 a orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.

VIII. ASSAY PROCEDURE (continued)

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 wells 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. 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 (Cat. # EZRMI-13K)

Option A: For Samples with significant Serum Matrix Effect

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

Assay Procedure for Rat / Mouse Insulin ELISA kit (Cat. # EZRMI-13K)

Option B: For Samples without significant Serum Matrix Effect

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

IX. 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	QC 1	Sample									
H	1 ng/mL	QC 1	Etc.									

X. 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 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., 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.

XI. INTERPRETATION

A. Acceptance Criteria

1. 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.

2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.

3. The limit of sensitivity of this assay is 0.2 ng/mL (35 pM) insulin (10 µL sample size).

4. The appropriate range of this assay is 0.2 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.

XII. ASSAY CHARACTERISTICS

A. 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 µL sample size.

B. 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 120 nM

C. 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 Millipore 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.

XII. ASSAY CHARACTERISTICS (continued)

D. Dilutional Linearity:

Serum Sample #	Dilution Factor	Insulin Level		
		Observed (ng/mL)	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		104
	8x	3.92		133
Rat Serum # 1	--	4.22	**4.22**	**100**
	2x	3.80		90
	5x	3.55		84
	10x	4.70		111
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.

XII. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike and Recovery of Insulin in Serum Samples:

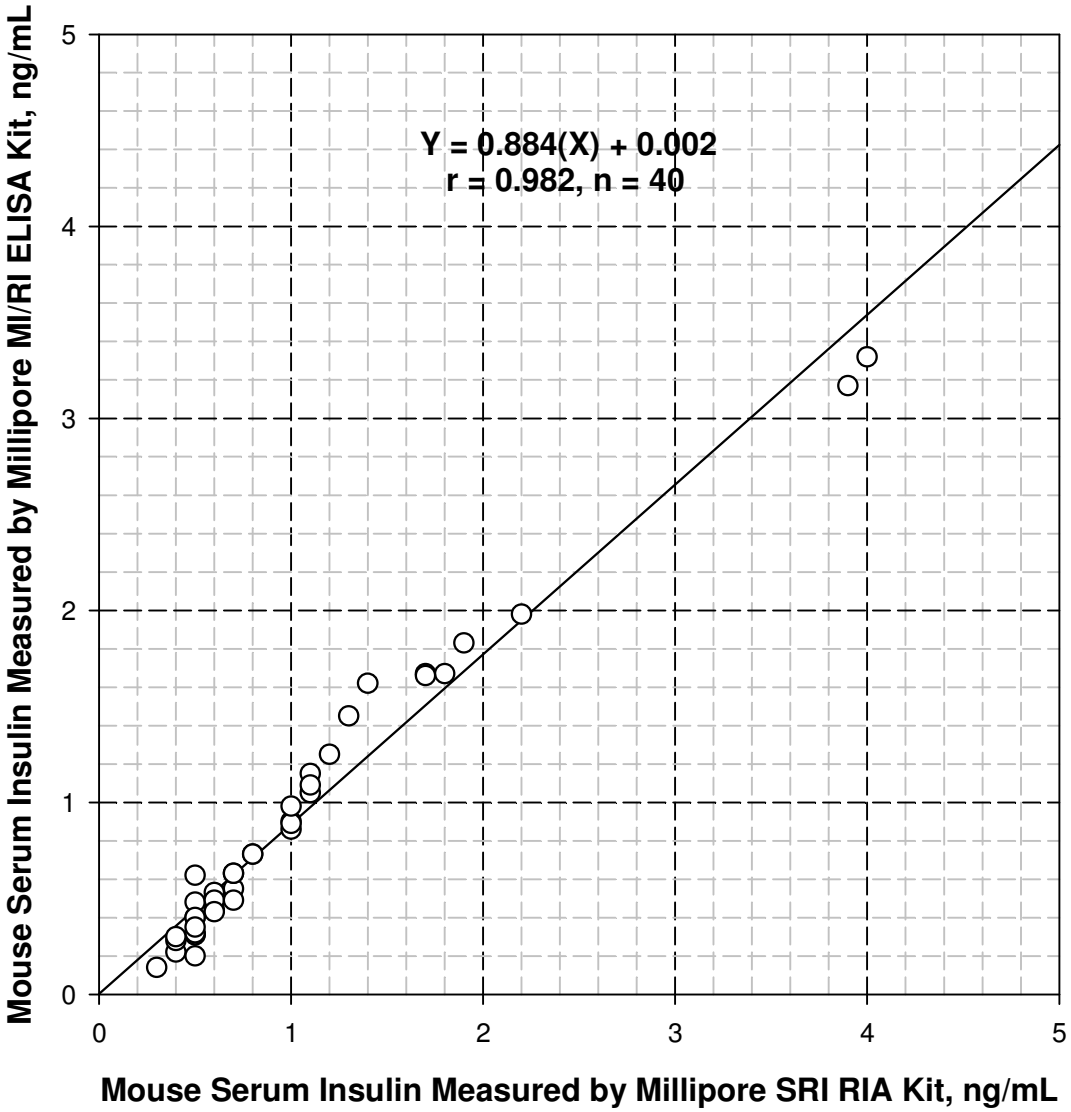
Serum Sample #	Rat Insulin		Recovery (%) of Spiked Insulin
	Added (ng/mL)	Observed (ng/mL)	
Mouse Serum # 1	0	0.33	**--**
	0.5	0.83	100
	2	2.15	91
	5	5.07	95
Mouse Serum # 2	0	1.78	**--**
	0.5	2.20	84
	2	3.43	83
	5	6.16	88
Mouse Serum # 3	0	1.01	**--**
	0.5	1.49	96
	2	2.91	95
	5	5.95	99
Rat Serum # 1	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.

XIII. CORRELATION GRAPH

Mouse Serum Insulin Assays:

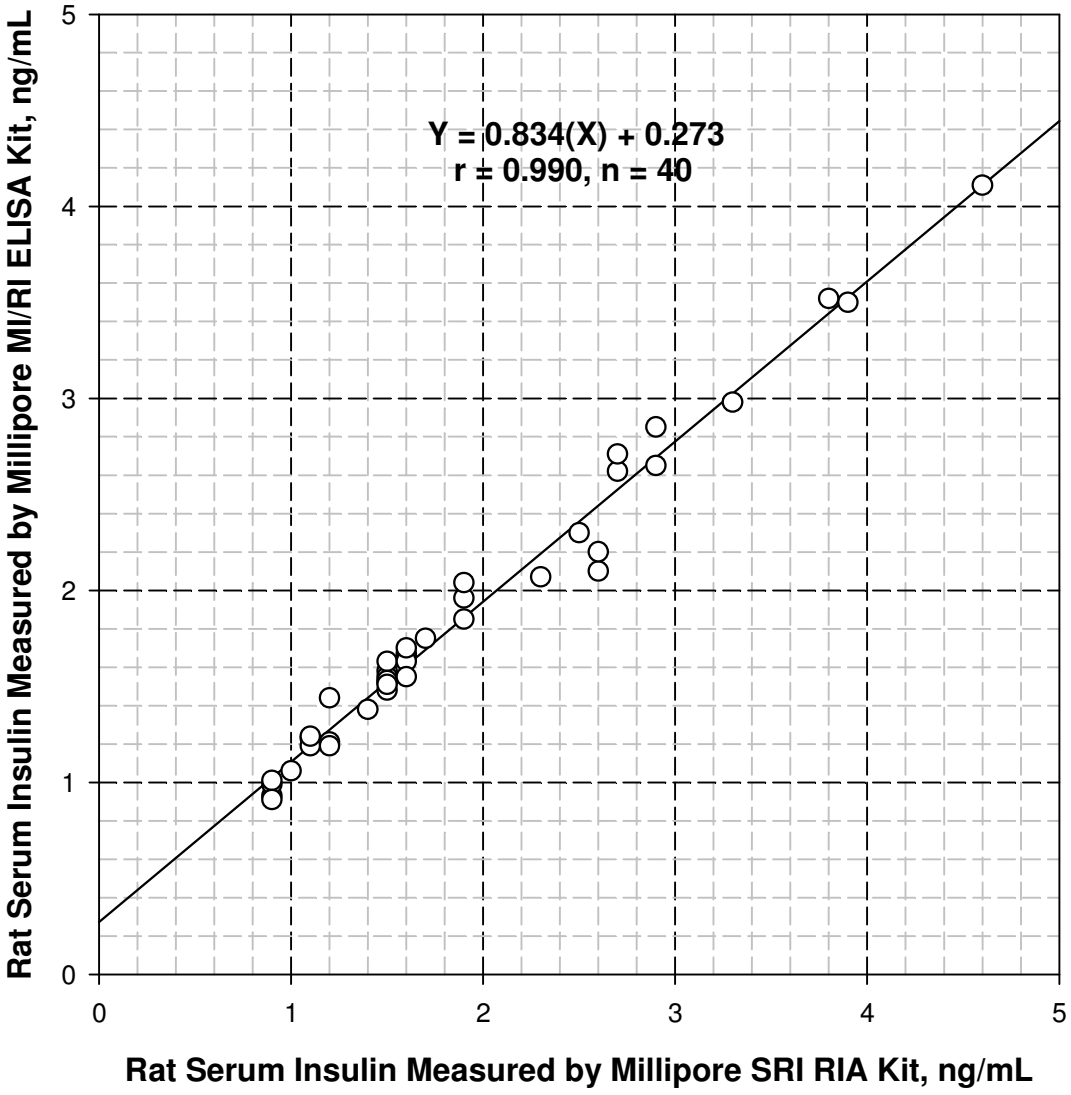
Correlation of Results by RIA and ELISA Methods



XIII. CORRELATION GRAPH (continued)

Rat Serum Insulin Assays:

Correlation of Results by RIA and ELISA Methods



XIV. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com.

XV. TROUBLE SHOOTING GUIDE

1. 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.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. 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.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure that all reagents and samples are added to the bottom of each well.
6. 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.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with TBS.

XVI. REPLACEMENT REAGENTS

REAGENTS	CAT. #
Rat/Mouse Insulin ELISA Plate	EP13
10X HRP Wash Buffer Concentrate (50 mL)	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-4
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XVII. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

TELEPHONE ORDERS:
Toll Free US (800) MILLIPORE
FAX ORDERS: (636) 441-8050
MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:
To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.