Human Insulin

96-Well Plate

Cat. # EZHI-14K

HUMAN INSULIN ELISA KIT
96-Well Plate (Cat. # EZHI-14K)

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I. INTENDED USE

This Human Insulin ELISA kit is used for the non-radioactive quantification of human insulin in serum, plasma and other biological media. This kit has no cross reactivity to intact human proinsulin and its major processed intermediate, des(31,32) proinsulin; however there can be crossreactivity with the minor intermediate, des(64,65) proinsulin, in serum. One kit is sufficient to measure 38 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 human insulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of monoclonal mouse anti-human insulin antibodies and the binding of a second biotinylated monoclonal mouse anti-human antibody to the captured insulin, 2) wash away of unbound materials from samples, 3) conjugation 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 after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human 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 human insulin.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

A. Human Insulin ELISA Plate
   Coated with Mouse Monoclonal anti-Human 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: 50 mL
   Preparation: Dilute 1:10 with deionized water

D. ELISA Human Insulin Standards
   Human Insulin in Buffer: 2, 5 10, 20, 50, 100, and 200 µU/mL
   Quantity: 0.5mL/bottle
   Preparation: Ready to Use

III. REAGENTS SUPPLIED (continued)

E. ELISA Quality Controls 1 and 2
Purified Recombinant Human Insulin in Assay Buffer
Quantity: 0.5mL/bottle
Preparation: Ready to Use

F. Matrix Solution
Heat-treated Charcoal Stripped Off the Clot Human Serum
Quantity: 1 mL/vial
Preparation: Ready to Use

G. Assay Buffer
0.05 M PBS, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium Azide, and 1% BSA
Quantity: 8 mL/vial
Preparation: Ready to Use

H. Human Insulin Detection Antibody
Pre-titered Biotinylated Monoclonal Mouse anti-Human Insulin Antibody
Quantity: 3 mL/vial
Preparation: Ready to Use

I. Enzyme Solution
Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
Quantity: 12 mL/vial
Preparation: Ready to Use

J. Substrate (TMB)
3, 3',5,5'-tetramethylbenzidine in Buffer
Quantity: 12 mL/vial
Preparation: Ready to use. Minimize the exposure to light.

K. ELISA Stop Solution
0.3 M HCl
Quantity: 12 mL/vial
Preparation: Ready to Use
[Caution: Corrosive Solution]

IV. STORAGE AND STABILITY

Upon receipt, all components of the kit should be stored at 2-8°C. For longer storage, freeze diluted HRP Wash Buffer, Matrix Solution, Insulin Standards and Controls at ≤ –20°C. Avoid multiple freeze/thaw cycles of the Insulin Standards 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 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.

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 eyes. Do not swallow or ingest.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and Pipette Tips: 10µL - 20 µL or 20µL - 100 µL
2. Multi-Channel Pipettes and Pipette Tips: 5 ~ 50 µL and 50 ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. To prepare serum samples, whole blood is directly drawn into a Vacutainer® serum tube that contains no anticoagulant. 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 store samples in aliquots at ≤ –20°C for later use. Avoid freeze/thaw cycles.

5. To prepare plasma samples, whole blood should be collected into Vacutainer® EDTA-plasma tubes and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

6. If heparin is to be used as an anticoagulant, 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.

VIII. ASSAY PROCEDURE

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

1. Dilute the 10X concentrated HRP Wash Buffer 10 fold by mixing the entire content of buffer with 450 mL deionized or glass distilled 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 and fill each well with 300 µl of diluted HRP Wash Buffer. Incubate at room temperature for 5 minutes. 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 an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.

3. Add 20 µL Assay Buffer to the NSB (Non-Specific Binding) wells and each of the sample wells (refer to IX for suggested well orientations).

4. If samples to be assayed are serum or plasma, add 20 µL Matrix Solution to the NSB, Standard, and Control wells. If samples are free of significant serum matrix components, add 20 µL Assay Buffer instead.

5. Add in duplicate 20 µL Human Insulin Standards in the order of ascending concentration to the appropriate wells.

7. Add 20 µL QC1 and 20 µL QC2 to the appropriate wells.

8. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells.

9. Add 20 µL Detection Antibody to all wells. **For best result all additions should be completed within 30 minutes.** Cover the plate with plate sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm).

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

10. Wash wells 3 times with diluted HRP 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 the plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

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

13. Wash wells 5 times with diluted HRP 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 on 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 monitor color development using 370 nm filter, if available on the spectrophotometer. When the absorbance is between 1.2 and 1.8 at 370 nm, the stop solution can be added to terminate the color development.

VIII. ASSAY PROCEDURE (continued)

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 to yellow after acidification. Read absorbance at 450 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well. The absorbance of highest insulin standard should be approximately 1.8 ~ 2.6.

Assay Procedure for Human Insulin ELISA kit (Cat. # EZHI-14K)

	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 10X Wash Buffer with 450mL Deionized Water.	Add 300 µL Wash Buffer to plate and incubate at room temperature for 5 minutes. Remove residual buffer by tapping smartly on absorbent towels.	Assay Buffer	Matrix Solution*	Standards/ Controls/ Samples	Detection Antibody	Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µl Wash Buffer	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 5X with 300 µL Wash Buffer	Substrate	Seal, Agitate, Incubate approximately 5-20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm.
A1, B1			20 µL	20 µL	----------	20 µL		100 µL		100 µL		100 µL	
C1, D1			------	20 µL	20 µL of 2 µU/mL Standard	20 µL		100 µL		100 µL		100 µL	
E1, F1			------	20 µL	20 µL of 5 µU/mL Standard	20 µL		100 µL		100 µL		100 µL	
G1, H1			------	20 µL	20 µL of 10 µU/mL Standard	20 µL		100 µL		100 µL		100 µL	
A2, B2			------	20 µL	20 µL of 20 µU/mL Standard	20 µL		100 µL		100 µL		100 µL	
C2, D2			------	20 µL	20 µL of 50 µU/mL	20 µL		100 µL		100 µL		100 µL	
E2, F2			------	20 µL	20 µL of 100 µU/mL Standard	20 µL		100 µL		100 µL		100 µL	
G2, H2			------	20 µL	20 µL of 200 µU/mL Standard	20 µL		100 µL		100 µL		100 µL	
A3, B3			------	20 µL	20 µL of QC 1	20 µL		100 µL		100 µL		100 µL	
C3, D3			------	20 µL	20 µL of QC 2	20 µL		100 µL		100 µL		100 µL	
E3, F3 ↓			20 µL	------	20 µL of Sample	20 µL		100 µL		100 µL		100 µL	

* See Section VIII. Assay Procedure Step 4: If samples are free of significant matrix components, add 20 µL Assay Buffer instead.

IX. MICROTITER PLATE ARRANGEMENT

Standard Human Insulin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	20 µU/mL	QC 1	Etc....								
B	NSB	20 µU/mL	QC 1									
C	2 µU/mL	50 µU/mL	QC 2									
D	2 µU/mL	50 µU/mL	QC 2									
E	5 µU/mL	100 µU/mL	Sample 1									
F	5 µU/mL	100 µU/mL	Sample 1									
G	10 µU/mL	200 µU/mL	Sample 2									
H	10 µU/mL	200 µU/mL	Sample 2									

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.

Note: When sample volumes assayed differ from 20 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µL, compensate the volume deficit with either matrix solution or assay buffer, whichever is appropriate.

XI. INTERPRETATION

1. The run 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 the supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.
3. The limit of sensitivity of this assay is 2 µU/mL human insulin (20 µl sample size).
4. The appropriate range of this assay is 2 µU/mL to 200 µU/mL human insulin (20 µl sample size). Any result greater than 200 µU/mL in a 20 µl sample should be repeated on dilution using either matrix solution or assay buffer as diluent, whichever is appropriate, until the results fall within range.

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Insulin that can be detected by this assay is 2 µU/mL when using a 20 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.

Human Insulin	100% [ED(50) = 0.68 nM]
Porcine Insulin	154%
Bovine Insulin	56%
Ovine Insulin	39%
Rat Insulin	n.d.*
Human Proinsulin	n.d.**
Des(64,65) Human Proinsulin	117%
Des(31,32) Human Proinsulin	0.3%
Porcine Proinsulin	< 0.1%
Bovine Proinsulin	< 0.1%
Human IGF-I	n.d.*
Human IGF-II	n.d.*
Glucagon	n.d.*
Glucagon-like Peptide 1	n.d.**
Human C-peptide	n.d.*
Rat C-peptide	n.d.*
Human Leptin	n.d.*
Rat Leptin	n.d.*
Mouse Leptin	n.d.*

n.d.: not detectable at concentrations up to * - 120 nM; and ** - 100 nM.

XII. ASSAY CHARACTERISTICS (continued)

C. Precision

Within and Between Assay Variation

Sample Number	Mean Insulin Level (µU/mL)	Assay Variation (% CV)	
		Intra-assay	Mean ± S.D.
1	7.0	6.94	5.96 ± 1.17
2	12.6	6.95	
3	94.8	5.29	
4	120.5	4.64	
		Inter-assay	Mean ± S.D
5	6.2	10.2	10.3 ± 0.9
6	64.4	11.4	
7	84.1	10.5	
8	120.9	9.1	

The assay variation of Millipore Human Insulin ELISA kit was studied on eight human serum samples with varying concentrations of endogenous analyte. The intra-assay variation of four samples was calculated from six duplicate determinations in an assay. The inter-assay variation was calculated from results of six separate assays with duplicate samples in each assay.

XII. ASSAY CHARACTERISTICS (continued)

D. Recovery

Spike & Recovery of Insulin in Human Serum

Serum Sample #	Insulin Added (µU/mL)	Mean Insulin Level (n = 3 assays)	
		Observed (µU/mL)	% of Recovery
1	0	3.0	**100**
	10	11.4	84
	50	42.8	80
	100	78.7	76
2	0	5.7	**100**
	10	14.5	88
	50	49.9	88
	100	87.7	82
3	0	6.6	**100**
	10	16.8	102
	50	53.2	93
	100	98.6	92
4	0	15.0	**100**
	10	25.4	103
	50	64.6	99
	100	111.6	97

Human insulin at indicated levels was added to four human serum samples and the resulting insulin content of each sample was determined in three separate assays. 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 10, 50 and 100 µU/mL is 94 ± 10%, 90 ± 8% and 87 ± 9%, respectively.

XII. ASSAY CHARACTERISTICS (continued)

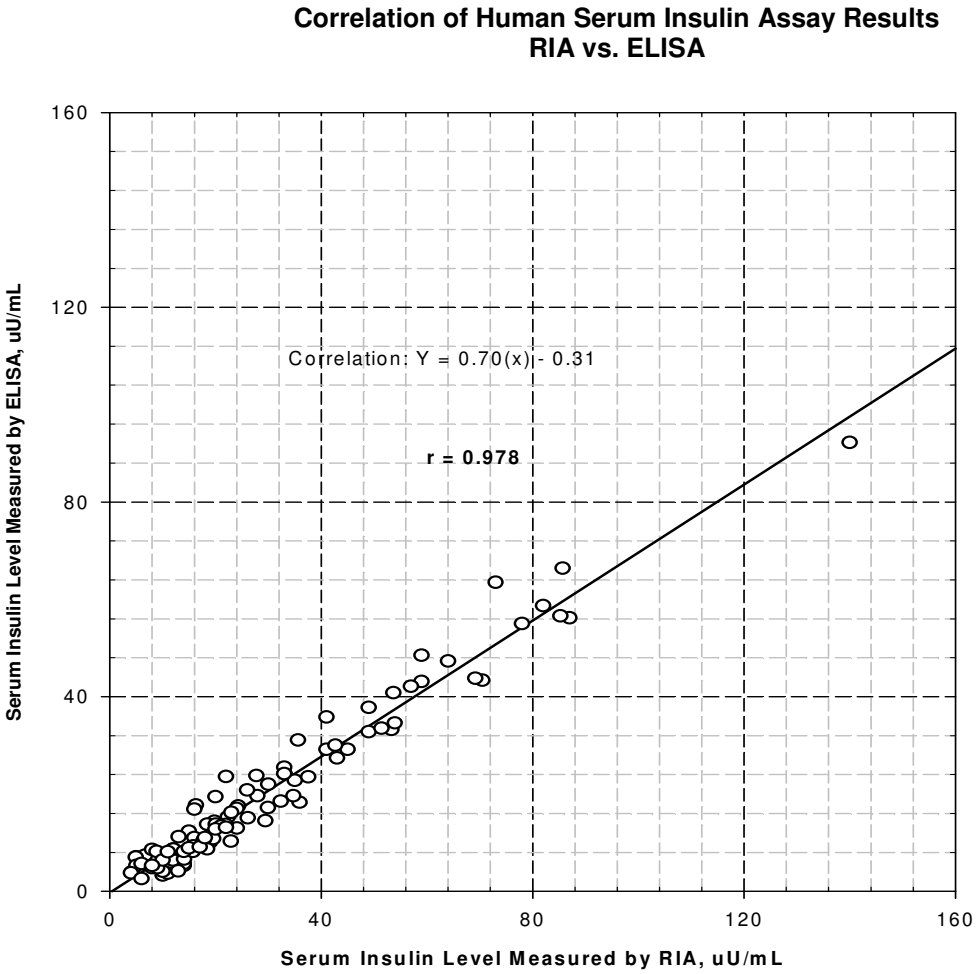
E. Linearity

Effect of Serum Dilution

Serum Sample #	Volume Assayed	Mean Insulin Level		
		Observed (µU/mL)	Expected (µU/mL)	% of Expected
1	20 µL	91.8	91.8	**100**
	15 µL	98.5		107
	10 µL	102.0		111
	5 µL	103.1		112
2	20 µL	118.6	118.6	**100**
	15 µL	118.6		100
	10 µL	120.2		101
	5 µL	127.1		107
3	20 µL	72.9	72.9	**100**
	15 µL	73.1		100
	10 µL	70.3		96
	5 µL	76.2		105

Three human serum samples with the indicated sample volumes were assayed in four separate experiments. Required amount of matrix solution was added to compensate for lost volumes below 20 µL. The resulting dilution factors of 1.0, 1.33, 2.0, and 4.0 representing 20 µL, 15 µL, 10 µL, and 5µL sample volumes assayed, respectively, were applied in the calculation of observed insulin concentrations. % of expected = observed/expected x 100%. Mean ± S.D. for the % of expected values at 15, 10 and 5 µL sample volumes assayed is 103 ± 3.6%, 103 ± 7.6% and 109 ± 3.0%, respectively.

XIII. CORRELATION GRAPH



Serum samples obtained from 97 human subjects were assayed for insulin content using both Millipore Human Insulin Specific RIA Kit (Catalogue # HI-14K) and Human Insulin ELISA Kit (Catalogue # EZHI-14K). Correlation of the two kits is derived by linear regression analysis of paired results from each sample.

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. TROUBLESHOOTING 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 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. Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.
9. 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. #
Human Insulin ELISA Plate	EP14
10X HRP Wash Buffer Concentrate	EWB-HRP
ELISA Human Insulin Standards	E8014-K
ELISA Quality Controls 1 and 2	E6000-K
Matrix Solution	EMTX
Assay Buffer	EABIR-2
Human Insulin Detection Antibody	E1014
Enzyme Solution	EHRP-3
Substrate (TMB)	ESS-TMB
ELISA 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:

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B. Conditions of Sale

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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.