Human Insulin (Animal Serum Free)

96-Well Plate

Cat. # EZHIASF-14K

HUMAN INSULIN ELISA KIT (ANIMAL SERUM FREE)
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I. INTENDED USE

Unlike other commercially available insulin ELISA kits, this Human Insulin Animal Serum Free (ASF) ELISA kit does not utilize animal proteins and the monoclonal antibodies are also harvested from clones grown in culture medium devoid of any animal protein. This 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 human proinsulin and C-peptide. 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 insulin antibody and the binding of a second biotinylated monoclonal insulin antibody to capture insulin, 2) wash away of unbound materials from samples, 3) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away the 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 ASF ELISA Plate
Coated with Monoclonal Insulin Antibody
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: 2 sheets
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 1:10 with distilled or deionized water

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

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

F. Matrix Solution
Treated Human Serum
Quantity: 1.0 mL/vial
Preparation: Ready to Use

G. Assay Buffer
0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05% ProClin300, and 0.1% Human Serum Albumin
Quantity: 10 mL/vial
Preparation: Ready to Use

H. Human Insulin ASF Detection Antibody
Pre-titered Biotinylated Monoclonal Insulin Antibody
Quantity: 12 mL/vial
Preparation: Ready to Use

III. REAGENTS SUPPLIED (continued)

I. Enzyme Dilution Buffer
0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05% ProClin300, and 0.1% Human Serum Albumin
Quantity: 12 mL/vial
Preparation: Ready to Use

J. Concentrated Enzyme Solution
Concentrated Streptavidin-Horseradish Peroxidase Conjugate
Quantity: 0.5 mL/vial
Preparation: Just prior to use, dilute Enzyme Solution 40 fold by mixing 0.3 mL Enzyme Solution with 11.7 mL Enzyme Dilution Buffer.

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

L. 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 Wash Buffer, Matrix Solution, Insulin Standards and Controls at ≤ –20°C. Avoid multiple freeze/thaw cycles of the Insulin Standards and Matrix Solution. Unused microtiter 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. Human Serum Albumin

The Human Serum Albumin (HSA) used in the preparation of this product is made from human serum and has the potential for bloodborne pathogens; strict adherence to a Bloodborne Pathogen Exposure Control Plan must be followed. Do not get in eyes, on skin, on clothing. Personal protective equipment must be worn when handling this material. The area must be decontaminated with 10% bleach and alcohol after preparation.

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 Wash Buffer 10 fold by mixing the entire contents of both buffer bottles with 900 mL deionized or 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 strips in an empty plate holder and fill each well with 300 µL of diluted 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. Wash the plate one more time with 300 µL of Wash Buffer. 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 80 µL Assay Buffer to the blank wells and sample wells. Add 60 µL Assay Buffer to Standard wells, and QC1 and QC2 wells. (See plate well map for suggested well orientation).

4. If samples to be assayed are serum or plasma, add 20 µL Matrix Solution to the NSB, Standard, and Control (QC1 and QC2) wells (refer to IX for suggested well orientations). 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.

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

7. Add sequentially 20 µL of the unknown samples in duplicate 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 90 minutes on an orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm).

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

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

VIII. ASSAY PROCEDURE (continued)

10. Add 100 µL Human Insulin ASF Detection Antibody to each well. Cover the plate with sealer and incubate with moderate shaking at room temperature for 1 hour on the microtiter plate shaker.

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

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

13. Add 100 µL of 40 fold diluted Concentrated Enzyme Solution to each well. **IMPORTANT: Dilute the concentrated enzyme solution just prior to use. See Section III.-J for preparation of dilution.** Cover the plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

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

15. Wash wells 5 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

16. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 5-20 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 blue 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.

17. 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 1.7-3.0.

Assay Procedure for Human Insulin ELISA Kit (Animal Serum Free) (Cat. # EZHIASF-14K)

	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 7-9	Step 10	Step 10-12	Step 13	Step 13-15	Step 16	Step 16	Step 17	Step 17
Well #	Dilute both bottles of 10X Wash Buffer with 900mL 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. Wash one additional time with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	Assay Buffer	Matrix/ Assay Buffer	Standards/ Controls/ Samples	Seal, Agitate, Incubate 90 minutes at Room Temperature. Wash 3X with 300 µL Wash Buffer	Detection Ab	Seal, Agitate, Incubate 1 hour at Room Temperature. Remove residual buffer by tapping smartly on absorbent towels. 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 5 - 20 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, A2			80 µL	20µL	---------		100 µL		100 µL		100 µL		100 µL	
B1, B2			60 µL	20µL	20 µL of 2 µU/mL Standard									
C1, C2			60 µL	20µL	20 µL of 5 µU/mL Standard									
D1, D2			60 µL	20µL	20 µL of 10 µU/mL Standard									
E1, E2			60 µL	20µL	20 µL of 20 µU/mL Standard									
F1, F2			60 µL	20µL	20 µL of 50 µU/mL Standard									
G1, G2			60 µL	20µL	20 µL of 100 µU/mL Standard									
H1, H2			60 µL	20µL	20 µL of 200 µU/mL Standard									
A3, A4			60 µL	20µL	20 µL of QC I									
B3, B4			60 µL	20µL	20 µL of QC II									
C3, C4			80 µL	--------	20 µL of Sample									
D3, D4			80 µL	--------	20 µL of Sample									

IX. MICROTITER PLATE ARRANGEMENT

Human Insulin ELISA (Animal Serum Free)

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

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 0.73 uU/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%
Human Proinsulin	n.d.**
Human C-peptide	n.d.*

n.d.: not detectable at concentrations up to * - 20 ng/mL; and ** - 2 ng/mL

XII. ASSAY CHARACTERISTICS (continued)

C. Precision

Within and Between Assay Variation

Sample Number	Mean Insulin Level (µU/mL)	Assay Variation (% CV)	
		Intra-assay	Mean
1	9.3	1.7	1.8
2	43.1	1.9	
		Inter-assay	Mean
3	11.0	7.1	6.6
4	55.2	6.0	

The intra-assay variation of two samples were calculated from eight duplicate determinations in a single assay. The inter-assay variation was calculated from results of five separate assays with duplicate samples in each assay.

D. Recovery

Spike & Recovery of Insulin in Human Plasma

Plasma Sample #	Insulin Added (µU/mL)	Mean Insulin Level (n = 3 assays)	
		Estimated (µU/mL)	% of Recovery
1	0	3.0	100
	6.25	11.4	123.2
	12.5	19.7	127.1
	50	35.8	127.9
2	0	11.4	100
	6.25	19.0	107.6
	12.5	24.0	100.4
	50	34.6	95.1
3	0	6.5	100
	6.25	12.2	95.7
	12.5	17.1	90.0
	50	27.6	87.6

Human insulin at indicated levels was added to three plasma samples and the insulin levels of basal and spiked plasma samples were determined with this ELISA kit The % of recovery = Estimated insulin level *100 / Expected insulin level (basal + added). Mean ± S.D. of recovery rate at spiked insulin level of 6.25, 12.5 and 25 µU/mL is 108.9 ± 13.8%, 105.8 ± 19.1% and 103.5 ± 21.4 %, respectively.

XII. ASSAY CHARACTERISTICS (continued)

E. Linearity

Effect of Plasma Dilution

Plasma Sample #	Dilution Factor	Mean Insulin Level		
		Estimated (µU/mL)	Expected (µU/mL)	% of Expected
1	8	8.9	96.8	72.7
	4	19.4		80.2
	2	41.4		85.5
	1	96.8		100.0
2	8	9.8	85.9	91.3
	4	20.3		94.5
	2	41.7		97.1
	1	85.9		100.0
3	8	9.0	73.6	97.8
	4	18.2		98.9
	2	35.3		95.9
	1	73.6		100.0

Three human plasma samples were spiked with exogenous human insulin. The spiked samples were then diluted 2, 4 and 8 fold with assay matrix and then these diluted and originally spiked samples assayed with this ELISA kit. % of expected = (Estimated value x Dilution factor) x 100/expected value from undiluted spiked sample. Mean ± S.D. for the % of expected values at 8, 4 and 2 fold dilutions are 87.3 ± 13.0%, 91.2 ± 9.8% and 92.9 ± 6.4%, respectively.

XIII. 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/techlibrary/index.do using the catalog number as the keyword.

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XIV. 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 absorbancy 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.

XV. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Microtiter Plates	EP14ASF
10X HRP Wash Buffer Concentrate	EWB-HRP
Human Insulin Standards	E8014-KASF
Quality Controls 1 and 2	E6000-KASF
Matrix Solution	EMTX
Assay Buffer	AB-PASF
Enzyme Dilution Buffer	EDB-ASF
Human Insulin Detection Antibody	E1014ASF
Concentrated Enzyme Solution	EHRP-ASF
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XVI. 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 in placing an order or obtaining additional information about MILLIPLEX™ MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at customerserviceEU@Millipore.com.

Conditions of Sale

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

Material Safety Data Sheets (MSDS)

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