



HUMAN TOTAL PROINSULIN ELISA KIT
96-Well Plate (Cat. # EZHPI-15K)

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HUMAN TOTAL PROINSULIN ELISA KIT

96-Well Plate (Cat. # EZHPI-15K)

I. INTENDED USE

This Human Total Proinsulin ELISA kit is used for the non-radioactive quantification of human total proinsulin in serum and plasma. This kit has 100% cross reactivity to intact human proinsulin and its major processed intermediate, des(31,32) proinsulin, and 81% cross reactivity to its processed intermediate des(64,65) proinsulin in serum and plasma. Human Insulin (up to 200 μ U/mL) and Human C-Peptide (up to 10ng/mL) do not interfere with the assay result. One kit is sufficient to measure 38 unknown samples in duplicate. This kit has no cross reactivity to human insulin. ***This kit is for research purpose only.***

II. PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of human proinsulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of polyclonal guinea pig anti-human insulin antibodies, 2) wash away of unbound materials from samples, 3) binding of a second biotinylated monoclonal mouse anti-human antibody to the c-peptide section of the captured molecules, 4) wash away of unbound materials from samples, 5) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 6) wash away of free enzyme, and 7) 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 human total proinsulin 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 proinsulin.

III. REAGENTS SUPPLIED

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

A. Human Proinsulin ELISA Plate

Coated with pretitered guinea pig 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

III. REAGENTS SUPPLIED (continued)

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 deionized water

D. Human Proinsulin Standards

Human Proinsulin in Buffer: 2, 5, 10, 20, 50, 100 and 200 pM.

Quantity: 0.5mL/bottle

Preparation: Ready to Use

E. ELISA Quality Controls 1 and 2

Purified Recombinant Human Proinsulin in Assay Buffer.

Quantity: 0.5mL/bottle

Preparation: Ready to Use

F. Matrix Solution

Charcoal stripped Proinsulin Depleted Human Serum.

Quantity: 1 mL/vial

Preparation: Ready to Use

G. Assay Buffer

0.025 M Phosphosaline, pH 6.8, containing 0.025 M EDTA, 0.08% Sodium Azide, 1% BSA.

Quantity: 9 mL/vial

Preparation: Ready to Use

H. Human Total Proinsulin Detection Antibody

Pre-titered Biotinylated Monoclonal Mouse anti-Human C-Peptide antibody.

Quantity: 12 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

3, 3',5,5'-tetramethylbenzidine in Buffer.

Quantity: 12 mL/vial

Preparation: Ready to Use.

Minimize exposure to light.

K. Stop Solution

0.3 M HCl

Quantity: 12 mL/vial

Preparation: Ready to Use

Caution: Corrosive Solution

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 Assay Buffer, HRP Wash Buffer, Matrix Solution, Proinsulin Standards and Quality Controls at $\leq -20^{\circ}\text{C}$. Minimize repeated freeze and thaw of the Proinsulin Standards, Quality Controls and Matrix Solution. Unused strips from the Microtiter Assay Plate 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. Pipettes and Pipette Tips: 10 μL - 20 μL or 20 μL - 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 and 590nm
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 $4 \pm 2^{\circ}\text{C}$.
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 $\leq -20^{\circ}\text{C}$ for later use. Avoid repeated 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 Wash Buffer concentrate 10 fold by mixing the entire content of each bottle of Wash Buffer with 450 mL deionized 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 the strips in an empty plate holder fill each well with 300 µL of diluted HRP 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. Wash assay plate using this procedure 3 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 60 µL Assay Buffer to the Standard and Quality Control wells.
4. Add 80 µL Assay Buffer to each of the NSB and sample wells.
5. Add 20 µL Matrix Solution to the NSB, Standard, and Quality Control wells (refer to IX for suggested well orientations).
6. Add in duplicate 20 µL Human Proinsulin Standards in the order of ascending concentration to the appropriate wells.
7. Add in duplicate 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. **For best result all additions should be completed within one hour.** 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 firmly after each wash to remove residual buffer.
11. Transfer Detection Antibody solution to a reagent reservoir and add 100 µL of this solution to each well with a multi-channel pipette. 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).

VIII. ASSAY PROCEDURE (continued)

12. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.
13. Wash wells 3 times with diluted HRP Wash Buffer, 300 μ L per well per wash. Decant and tap firmly after each wash to remove residual buffer.
14. Add 100 μ L 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.
15. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.
16. Wash wells 6 times with diluted HRP Wash Buffer, 300 μ L per well per wash. Decant and tap firmly after each wash to remove residual buffer.
17. Add 100 μ L of substrate solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 15-20 minutes.

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.

18. Remove sealer and add 100 μ L 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 are no air bubbles in any well. If wavelength correction is available, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 590, 600, or 620 nm. If the absorbance readings exceed the limitations of the plate reader, a second reading at 405 nm is needed (reference filter 590, 600 or 620 if available). In this case, proceed to construct a second standard curve as above with the absorbance readings of all Standards at 405 nm. The concentrations of the off-scale samples at 450 nm are then read from the new standard curve. The readings at 405 nm should not replace the on-scale readings at 450 nm.

IX. MICROTITER PLATE ARRANGEMENT

Standard Human Proinsulin ELISA

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

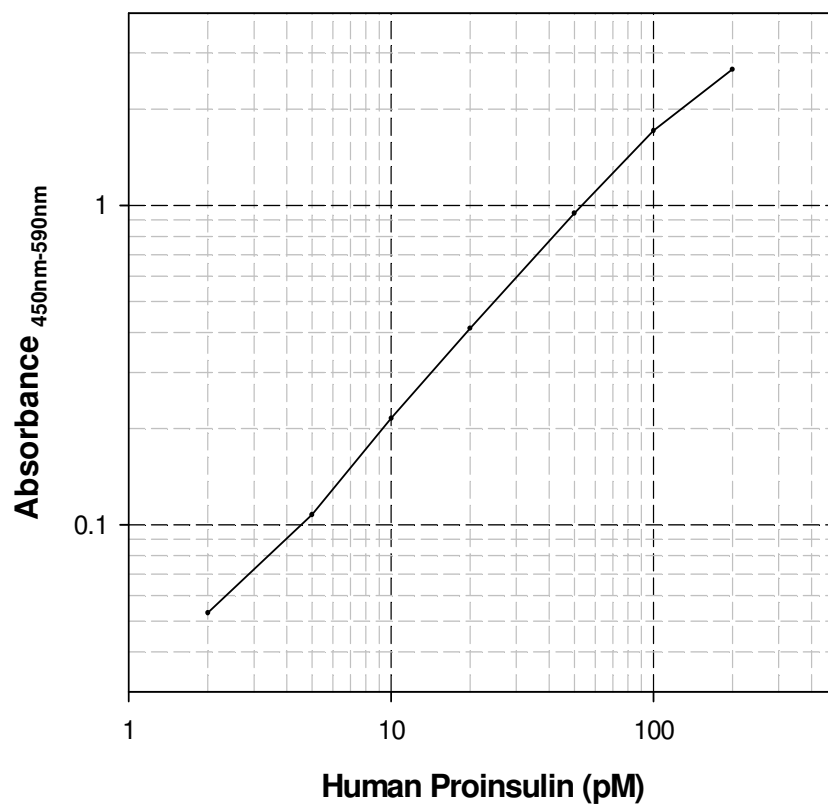
Note: When sample volumes assayed differ from 20 μL , an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 μL of sample is used, then observed data must be multiplied by 2). When sample volume assayed is less than 20 μL , compensate the volume deficit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

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 >15% CV, repeat the sample.
3. The limit of sensitivity of this assay is 0.5pM human proinsulin (20 μL sample size).
4. The dynamic range of this assay is 2 pM to 200 pM human proinsulin (20 μL sample size). Any result greater than 200 pM in a 20 μL sample should be diluted using matrix solution as diluent, and the assay repeated until the results fall within range.

XII. STANDARD CURVE AND CORRELATION GRAPH

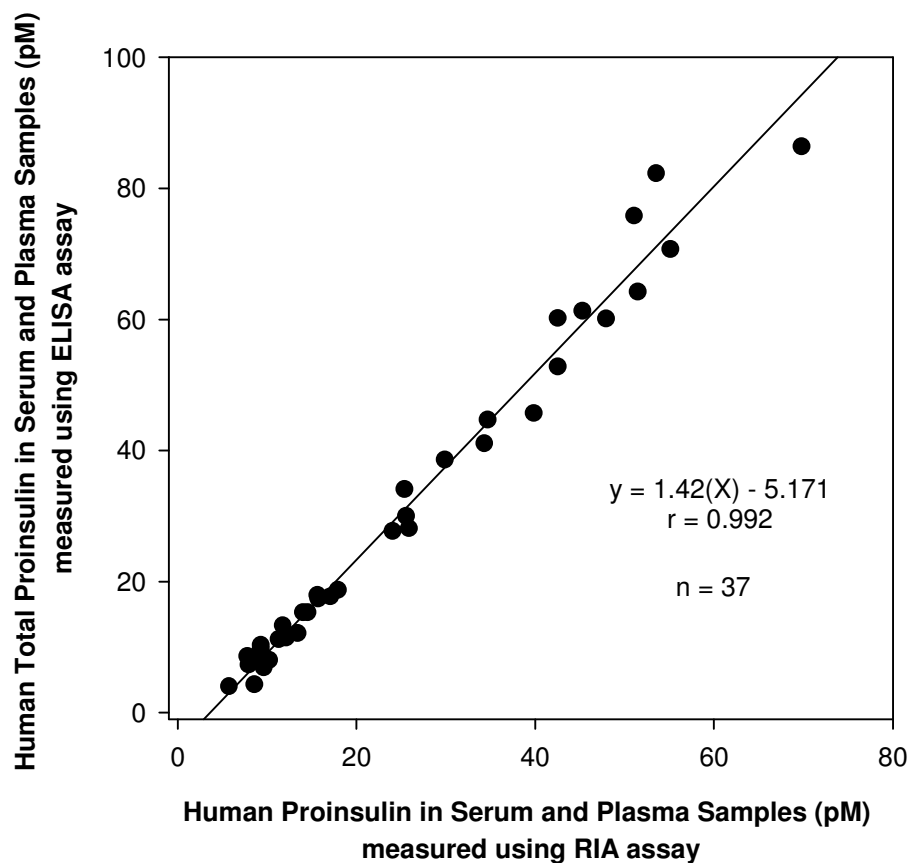
Human Proinsulin ELISA



For Reference Only – Typical Standard Curve

XII. STANDARD CURVE AND CORRELATION GRAPH (continued)

Human Proinsulin ELISA vs RIA Correlation



Serum samples obtained from 37 human subjects were assayed for proinsulin content using both Millipore Human Proinsulin RIA Kit (Catalogue #HPI-15K) and Human Total Proinsulin ELISA Kit (Catalogue #EZHPI-15K). Correlation of the two kits are derived by linear regression analysis of paired results from each sample.

XIII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Proinsulin that can be detected by this assay is 0.5 pM 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	n.d.*
Intact Human Proinsulin	100%
Des(64,65) Human Proinsulin	81%
Des(31,32) Human Proinsulin	100%
Porcine Proinsulin	~1%
Bovine Proinsulin	n.d.*
Glucagon	n.d.*
Human C-peptide	n.d.*
Rat C-peptide	n.d.*
Human Leptin	n.d.*
Rat Leptin	n.d.*

n.d.: not detectable at concentrations up to * 20 nM

C. Precision

Within and Between Assay Variation

Sample No.	Mean Proinsulin Levels (pM)	Within % CV	Between % CV
1	15.15	6.3	3.0
2	80.63	0.8	2.9
3	6.52	8.5	8.3

The assay variations of Millipore Human Total Proinsulin ELISA kits were studied on three human serum samples with varying concentrations of endogenous proinsulin. The mean within variation was calculated from results of six duplicate determinations in each assay of the indicated samples. The mean between variation of each sample was calculated from results of four separate assays with duplicate samples in each assay.

XIII. ASSAY CHARACTERISTICS (continued)

D. Recovery

Spike & Recovery of Human Proinsulin in Serum

Sample No.	Proinsulin Added (pM)	Expected (pM)	Observed (pM)	% of Recovery
1	0	16.1	16.1	100
	10	26.1	26.1	100
	50	66.1	63.7	96.4
	100	116.1	108.8	93.7
2	0	82.4	82.4	100
	10	92.4	91.4	98.9
	50	132.4	119.3	90.3
	100	182.4	160.9	88.2
3	0	7.5	7.5	100
	10	17.5	18	103
	50	57.5	56.3	97.9
	100	107.5	100.8	93.8

Varying amounts of human proinsulin were added to three human serum samples and the proinsulin content was determined in four separate assays.

The % of recovery = observed proinsulin concentrations/expected proinsulin concentrations x 100%.

XIII. ASSAY CHARACTERISTICS (continued)

E. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled	Expected (pM)	Observed (pM)	% Of Expected
1	20 µL	52.8	52.8	100
	10 µL		53.4	101
	4 µL		52.5	100
	2 µL		64	120
2	20 µL	85.9	85.9	100
	10 µL		86	100
	4 µL		81.5	95
	2 µL		82	95
3	20 µL	17.9	17.9	100
	10 µL		18.4	103
	4 µL		16	89
	2 µL		15	84

Three human serum samples with the indicated sample volumes were assayed in four separate experiments. Required amounts of assay buffer or matrix solution were added to compensate for lost volumes below 20 µL. The resulting dilution factors of 1.0, 2.0, 5.0, and 10.0 representing 20 µL, 10 µL, 4 µL, and 2 µL sample volumes assayed, respectively, were applied in the calculation of observed proinsulin concentrations.
 $\% \text{ expected} = \text{observed/expected} \times 100\%$.

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 HRP Wash Buffer or 3) overexposure to light after substrate has been added.

XVI. REPLACEMENT REAGENTS

Reagents

Human Proinsulin ELISA Plate
10X HRP Wash Buffer Concentrate (50 mL)
Human Proinsulin Standards
ELISA Quality Controls 1 and 2
Matrix Solution
Assay Buffer
Human Total Proinsulin Detection Antibody
Enzyme Solution
Substrate
Stop Solution

Cat. #

EP15
EWB-HRP
E8015-K
E6000-K
EMTX
EABU
E1015
EHRP
ESS-TMB
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 (866) 441-8400
(636) 441-8400

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.