INTACT HUMAN PROINSULIN ELISA KIT
96-Well Plate (Cat. # EZHIPI-17K)

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I. INTENDED USE

This Intact Proinsulin ELISA kit is used for the non-radioactive quantification of intact human proinsulin in serum and plasma. This kit does not cross-react with human insulin as high as 10 nM. In addition, presence of insulin (up to 208µU/ml) in serum or plasma does not interfere with the assay result. The kit has no cross-reactivity with the major species of proinsulin metabolites des(31,32) proinsulin while cross-reacts only weakly with the minor intermediate des(64,65) proinsulin. One kit is sufficient to measure 37 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 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 antibody specific to the B chain-C peptide junctional sequences 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'-tetra-methylbenzidine. 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

B. Adhesive Plate Sealer
Quantity: 2 sheets
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. Intact Proinsulin Standards

Human Proinsulin in Buffer: 0.5, 1, 2, 5, 10, 20, 50, and 100 pM.
Quantity: 0.5ml/bottle
Preparation: Ready to Use

E. Quality Controls 1 and 2

Purified Recombinant Human Proinsulin in Buffer.
Quantity: 0.5ml/bottle
Preparation: Ready to Use

F. Matrix Solution

Charcoal stripped pooled human Serum.
Quantity: 1.5 ml/vial
Preparation: Ready to Use

G. Assay Buffer

0.025 M Phosphate Buffer, pH 6.8, containing 0.025 M EDTA, 0.08% Sodium Azide, 1% BSA.
Quantity: 9 ml/vial
Preparation: Ready to Use

H. Human Intact Proinsulin Detection Antibody

Pre-titered biotinylated mouse monoclonal 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.

III. REAGENTS SUPPLIED (continued)

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°. For longer storage (> 2 weeks), freeze Assay Buffer, Wash Buffer, Matrix Solution, Proinsulin Standards and Quality Controls at ≤ –20°C. Minimize repeated freeze and thaw of the Proinsulin Standards, Quality Controls and Matrix Solution. 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: 20 µl ~ 100 µl
2. Multi-Channel Pipettes and Pipette Tips: 50 µl ~ 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. **Immediately add enough aprotinin to a final concentration of 500 KIU/ml**. Mix well and 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 repeated freeze/thaw cycles.

5. To prepare plasma samples, whole blood should be collected into Vacutainer® EDTA-plasma tubes and placed on ice. **Immediately add enough aprotinin to a final concentration of 500 KIU/ml**, mix well and centrifuge at 2,000 to 3,000 x g for 15 min at 4 ± 2°C. Observe the same precautions in the preparation of serum samples.

6. Other protease inhibitors or cocktails of inhibitors may be used instead of aprotinin, but the optimal concentrations to prevent degradation of intact proinsulin should be pre-determined.

7. 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.

8. 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 Microtiter Assay Plate from the foil pouch and fill each well with 300 µl of diluted 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 50 µl Matrix Solutions to Blank, Standards and Quality Control wells (refer to IX for suggested well orientations)..

4. Add 50 µl Assay Buffer to each of the Blank and sample wells.

5. Add in duplicate 50 µl Human Proinsulin Standards in the order of ascending concentration to the appropriate wells.

VIII. ASSAY PROCEDURE (continued)

6. Add in duplicate 50 µl QC1 and 50 µl QC2 to the appropriate wells.

7. Add sequentially 50 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).

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 firmly after each wash to remove residual buffer.

10. 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).

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

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

13. 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.

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

15. Wash wells 6 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap firmly after each wash to remove residual buffer.

16. Add 100 µl of Substrate Solution to each well, cover plate with sealer and shake in the plate shaker for 15 ~ 25 minutes. Blue color should be formed in wells of reference standards with intensity proportional to increasing concentrations of proinsulin. 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. Alternatively, the increase in blue color can be monitored at 630 nm wavelength and the stop solution to be added when the absorbance of the highest standard wells reached 0.8 to 0.9. The optimal time for substrate incubation may vary among laboratories.

Assay Procedure for Intact Human Proinsulin ELISA kit (Cat. # EZHIPI-17K)

	Step 1	Step 2	Step 3	Step4	Step 5-7	Step 7-9	Step 10	Step 10-12	Step 13	Step 13-15	Step 16	Step 16	Step 16	Step 16
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**	Matrix Solution	Assay Buffer	Standards/ Controls/ Samples	**Seal, Agitate, Incubate 1 hour at Room Temperature. Wash 3X with 300 µl Wash Buffer**	Detection Ab	**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 6X with 300 µl Wash Buffer**	Substrate	**Seal, Agitate, Incubate 15 - 25 minutes at Room Temperature.**	Stop Solution	**Read Absorbance at 450 nm and 590 nm.**
A1, A2			50 µl	50 µl			100 µl		100 µl		100 µl		100 µl	
A3, A4			50 µl		50 µl of 0.5 pM Standard									
A5, A6			50 µl		50 µl of 1 pM Standard									
A7, A8			50 µl		50 µl of 2 pM Standard									
A9, A10			50 µl		50 µl of 5 pM Standard									
A11, A12			50 µl		50 µl of 10 pM Standard									
B1, B2			50 µl		50 µl of 20 pM Standard									
B3, B4			50 µl		50 µl of 50 pM Standard									
B5, B6			50 µl		50 µl of 100 pM Standard									
B7, B8			50 µl		50 µl of QC I									
B9, B10			50 µl		50 µl of QC II									
B11, B12			----------	50 µl	50 µl of Sample									
C1, C2 ↓				50 µl	50 µl of Sample									

IX. MICROTITER PLATE ARRANGEMENT

Standard Intact Human Proinsulin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	0.5 pM	0.5 pM	1 pM	1 pM	2 pM	2 pM	5 pM	5 pM	10 pM	10 pM
B	20 pM	20 pM	50 pM	50 pM	100 pM	100 pM	QC 1	QC 1	QC 2	QC 2	Sample 1	Sample 1
C	Sample 2	Sample 2	Etc.	Etc.								
D												
E												
F												
G												
H												

X. CALCULATIONS

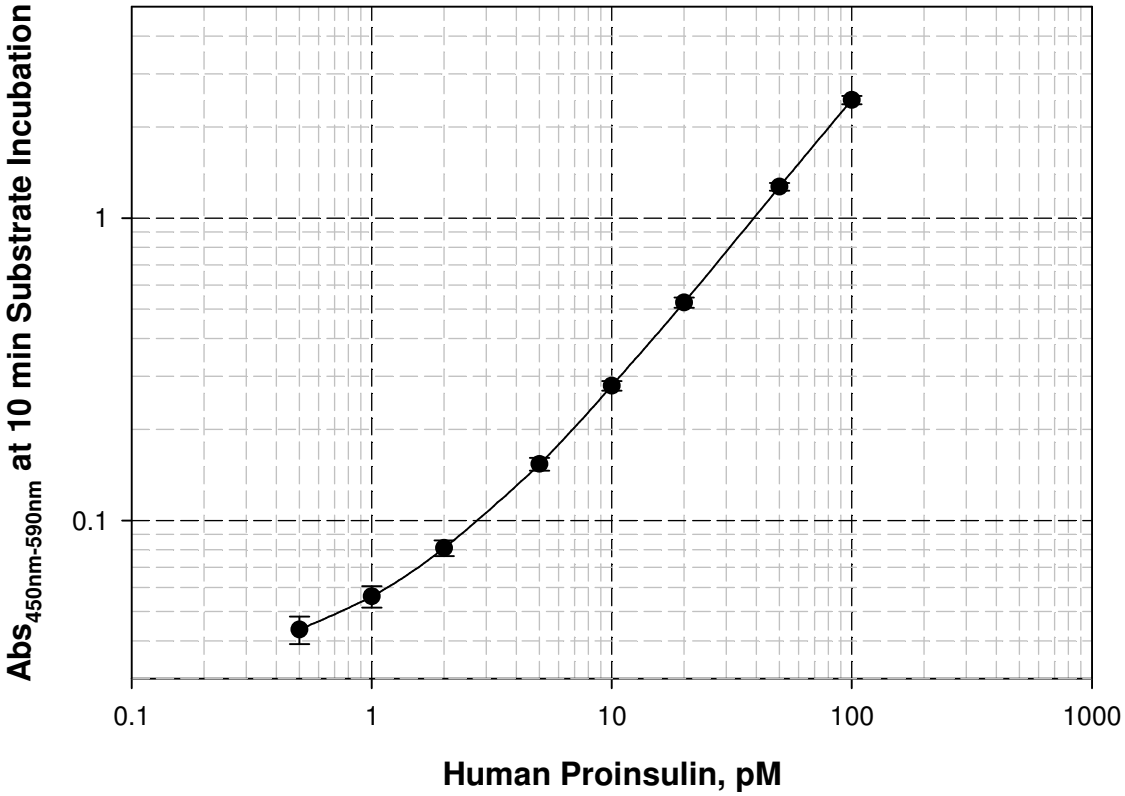
Graph a reference curve by plotting the absorbance unit of 450nm, less unit at 590nm, on the Y-axis against the concentrations of proinsulin standard on the X-axis The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Note: When sample volumes assayed differ from 50 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 25 µl of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 50 µl, compensate the volume deficit with either matrix solution or assay buffer, whichever is appropriate.

XI. INTERPRETATION

1. The assay should be rejected if one of the two QCs falls outside of 2 standard deviations of the applicable mean. See 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 0.1 pM human proinsulin (50 µl sample size).
4. The dynamic range of this assay is 0.5 pM to 100 pM human proinsulin (50 µl sample size). Any result greater than 100 pM in a 50 µl sample should be diluted using matrix solution or assay buffer as diluent, whichever is appropriate, and the assay repeated until the results fall within range.

XII. Graph of Typical Reference Curve (n = 11 assays)



For Demonstration Only – Do not use for calculations

XIII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Intact proinsulin that can be detected by this assay is 0.1 pM when using a 50 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.

Intact Human Proinsulin	100%
Des(64,65) Human Proinsulin	36%
Des(31,32) Human Proinsulin	n.d.
Bovine Proinsulin	n.d.
Porcine Proinsulin	n.d.
Human Insulin	n.d.
Bovine Insulin	n.d.
Ovine Insulin	n.d.
Porcine Insulin	n.d.
Rat Insulin	n.d.

n.d.: not detectable

C. Precision

Within and Between Assay Variations

Sample No.	Mean Intact Proinsulin Levels (pM)	Within % CV	Between % CV
1	1.41	7.7	6.4
2	12.2	0.4	3.3
3	29.1	0.4	9.0

The assay variations of Linco Human Intact Proinsulin ELISA kits were studied on three pooled human serum samples with varying concentrations of endogenous proinsulin. Within assay variations (intra-assay) were calculated from results of six duplicate determinations in one assay. Between assay variations (inter-assay) were calculated from results of six separate assays with duplicate samples in each assay.

XIII. ASSAY CHARACTERISTICS (continued)

D. Spike Recovery Rate of Intact Human Proinsulin in Assay Samples

Sample No.	Proinsulin Added (pM)	Expected (pM)	Observed (pM)	% of Recovery
Serum 1	0	2.20	2.20	100
	4	6.20	5.84	94.2
	40	42.20	39.82	94.3
	80	82.20	78.08	95.0
Serum 2	0	3.95	3.95	100
	4	7.95	7.61	95.7
	40	43.95	41.33	94.0
	80	83.95	78.36	93.3
Serum 3	0	1.57	1.57	100
	4	5.57	5.20	93.4
	40	41.57	38.52	92.7
	80	81.57	75.94	93.1
Plasma 1	0	2.61	2.61	100
	4	6.61	6.25	94.6
	40	42.61	10.09	94.1
	80	82.61	77.54	93.9
Plasma 2	0	4.37	4.37	100
	4	8.37	7.99	95.5
	40	43.37	42.01	94.7
	80	83.37	80.32	95.2
Plasma 3	0	2.02	2.02	100
	4	6.02	5.84	97.0
	40	42.02	39.44	93.9
	80	82.02	76.52	93.3

Varying amounts of human proinsulin were added to three human serum and plasma samples and the intact proinsulin content was assayed with Intact Human Proinsulin ELISA method. The % of recovery = observed proinsulin concentrations/expected proinsulin concentrations x 100%.

XIII. ASSAY CHARACTERISTICS (continued)

E. Linearity of Sample Dilution

Sample No.	Volume Sampled	Expected (pM)	Observed (pM)	% Of Expected
Serum 1	50µl	11.93	11.93	100
	30µl		11.92	99.9
	20µl		11.88	99.5
	10µl		10.90	91.4
Serum 2	50µl	19.46	19.46	100
	30µl		19.35	99.4
	20µl		19.28	99.0
	10µl		19.40	99.7
Serum 3	50µl	14.38	14.38	100
	30µl		14.30	99.4
	20µl		14.25	99.1
	10µl		13.60	94.6
Plasma 1	50µl	10.03	10.03	100
	30µl		10.42	103.9
	20µl		10.13	101.0
	10µl		10.10	100.7
Plasma 2	50µl	18.40	18.40	100
	30µl		18.38	99.9
	20µl		18.10	98.4
	10µl		18.15	98.6
Plasma 3	50µl	13.23	13.23	100
	30µl		12.97	98.0
	20µl		12.60	95.2
	10µl		12.80	96.7

Three human serum and plasma samples each with the indicated sample volumes were assayed with Intact Human proinsulin ELISA method. Required amounts of matrix solution were added to compensate for lost volumes below 50 µl. The resulting dilution factors of 1.0, 1.67, 2.5, and 5.0 representing 50 µl, 30 µl, 20 µl, and 10 µl sample volumes assayed, respectively, were applied in the calculation of observed proinsulin concentrations. % expected = observed/expected x 100%.

XIV. QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Linco Research website www.lincoresearch.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 Wash Buffer or 3) overexposure to light after substrate has been added.

XVI. REPLACEMENT REAGENTS

Reagents	**Cat. #**
Human Proinsulin ELISA Plates	EP15
10X HRP Wash Buffer Concentrate (50 ml)	EWB-HRP
Intact Proinsulin Standards	E8017-K
Quality Controls 1 and 2	E6000-K
Matrix Solution	EMTX-U
Assay Buffer	EABU
Human Intact Proinsulin Detection Antibody	E1017
Enzyme Solution	EHRP
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 (866) 441-8400
(636) 441-8400
FAX ORDERS: (636) 441-8050
MAIL ORDERS: Linco Research
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St. Charles, Missouri 63304 U.S.A.

For International Customers:
To best serve our international customers, it is LINCO's policy to sell our products through a network of distributors. To place an order or to obtain additional information about LINCO 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 Linco Research products may be ordered by fax or phone. See Section A above for details on ordering.