HUMAN PROINSULIN RIA KIT
250 TUBES (Cat. # HPI-15K)

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I. INTENDED USE

This Human Proinsulin Kit is for the quantitative determination of Proinsulin in serum, plasma, and other biological media. This assay cross-reacts neither with Human Insulin (<0.1%) nor with Human C-Peptide (0.1%) and therefore measures "true" Proinsulin levels. It is a completely homologous assay since the antibody was raised against purified Human Proinsulin and both the standard and the tracer are prepared with Human Proinsulin. ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The LINCO Research Human Proinsulin assay utilizes ^{125}I-labeled Human Proinsulin and a Human Proinsulin antiserum to determine the level of Proinsulin in serum, plasma or tissue culture media by the double antibody/PEG technique.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 250 tubes and contains the following reagents:

A. Assay Buffer
0.05M Phosphosaline pH 7.4 containing 0.025M EDTA, 0.08% Sodium Azide, and 1% RIA Grade BSA
Quantity: 40 ml/vial
Preparation: Ready to use

B. Human Proinsulin Antibody
Goat anti-Human Proinsulin Specific Antibody Serum in Assay Buffer
Quantity: 26 ml/vial
Preparation: Ready to use

C. ^{125} I-Human Proinsulin
^{125} I-Proinsulin Label, HPLC purified (specific activity 229 µCi/µg)
Lyophilized for stability. Freshly iodinated label contains <3 µCi, (111 kBq) calibrated to the 1st Monday of each month.
Quantity: 27 ml/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with entire contents of Label Hydrating Buffer. Allow to sit at room temperature for 30 minutes, with occasional gentle mixing.

D. Human Proinsulin Label Hydrating Buffer
Assay Buffer containing Normal Goat Serum as a carrier. Used to hydrate ^{125}I-Proinsulin.
Quantity: 27 ml/vial
Preparation: Ready to use

III. REAGENTS SUPPLIED (continued)

E. Human Proinsulin Standards
Purified Recombinant Human Proinsulin in Assay Buffer at the following concentrations:
2, 5, 10, 20, 50, 100 pM
Quantity: 2 ml/vial
Preparation: Ready to use

F. Matrix Solution
For correction of matrix effect in serum and plasma samples. Sample contains 0.08% Sodium Azide
Quantity: 6 ml/bottle

G. Quality Controls 1 & 2
Purified Recombinant Human Proinsulin in Assay Buffer
Quantity: 1 ml/vial
Preparation: Ready to use

H. Precipitating Reagent
Donkey anti Goat IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide
Quantity: 260 ml/vial
Preparation: Ready to use; chill to 4°C.

IV. STORAGE AND STABILITY

Refrigerate all reagents between 2 and 8°C for short term storage. For prolonged storage (>2 weeks), freeze at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles. Refer to date on bottle for expiration when stored at ≤ -20°C. Do not mix reagents from different kits unless they have the same lot number.

V. REAGENT PRECAUTIONS

A. Radioactive Materials
This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission (NRC) or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

The following are suggested general rules for the safe use of radioactive material. The customer's Radiation Safety Officer is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal protective devices at all times while in areas where radioactive materials are used or stored.

2. Wear laboratory coats, disposable gloves, and other protective clothing at all times.

3. Monitor hands, shoes, and clothing and immediate area surrounding the work station for contamination after each procedure and before leaving the area.

4. Do not eat, drink, or smoke in any area where radioactive materials are stored or used.

5. Never pipette radioactive material by mouth.

6. Dispose of radioactive waste in accordance with NRC rules and regulations.

7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.

V. REAGENT PRECAUTIONS (continued)

8. Use absorbent pads for containing and easily disposing of small amounts of contamination.

9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform Radiation Safety Officer.

B. Sodium Azide

Sodium Azide has been added to all 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.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may be used if the investigator finds that the pellet formation is acceptably stable in their system.)
2. 100 µl and 200 µl pipettes with disposable tips
3. 100 µl & 1.0 ml repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets are not recommended.)
5. Absorbent paper
6. Vortex mixer
7. Refrigerator
8. Gamma Counter

VII. SPECIMEN COLLECTION AND STORAGE

1. A maximum of 200 µl per assay tube of serum or plasma can be used. Smaller volumes of sample may be used when Proinsulin concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer must be added to compensate for the difference so the volume is equivalent to 200 µl, e.g., when using 100µl sample, add 100µl buffer. (See the note under the "Calculations" section.)
2. Care must be taken when using heparin as an anticoagulant, since an excess will provide falsely high values. Use no more than 10 IU heparin per ml of blood collected.
3. Specimens can be stored at 4°C if they will be tested within 24 hours of collection. For longer storage, specimens should be stored at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles.
4. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended.

A. Assay Set-Up, Day One

1. Pipet 400 µl of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 µl to Reference (Bo) tubes (5-6), no buffer to tubes 7-22, and 100 µl to tubes 23 through the end of the assay.
2. Pipet 200 µl of Standards and Quality Controls in duplicate (see flow chart).
3. Pipet 200 µl of each Sample in duplicate. (NOTE: Smaller volumes of sample may be used when Proinsulin concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 200 µl, e.g., when using 100 µl of sample, add 100 µl of Assay Buffer). Refer to Section IX for calculation modification.
4. Pipet 100 µl of Matrix Solution-HPI to tubes 5 to 22.
5. Pipet 100 µl of Human Proinsulin antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
6. Vortex, cover, and incubate 48 hours at room temperature.

VIII. ASSAY PROCEDURE (continued)

B. Day Three

7. Pipet 100 µl of hydrated ^{125}I-Proinsulin tracer to all tubes. Important: For preparation, see Section III, Part C.
8. Vortex, cover, and incubate 22 hours at room temperature.

C. Day Four

9. Add 1.0 ml of cold (4°C) Precipitating Reagent to all tubes (except Total Count tubes).
10. Vortex and incubate 20 minutes at 4°C.
11. Centrifuge, 4°C, all tubes [except Total Count tubes (1-2)] for 25 minutes at 2,000-3,000 xg. NOTE: If less than 2,000 xg is used or if slipped pellets have been observed in previous runs, the time of centrifugation must be increased to obtain a firm pellet (e.g., 40 minutes). Multiple centrifuge runs within an assay must be consistent.

    Conversion of rpm to xg:
    $xg = (1.12 \times 10^{-5})\ (r)\ (rpm)^2$
    r = radial distance in cm (from axis of rotation to the bottom of the tube)
    rpm = revolutions per minute

12. Immediately decant the supernatant of all tubes except Total Count tubes (1-2), drain tubes for at least 15-60 seconds (be consistent between racks), and blot excess liquid from lip of tubes. NOTE: Invert tubes only one time. Pellets are fragile and slipping may occur.
13. Count all tubes in a gamma counter for 1 minute. Calculate the pM of Human Proinsulin in unknown samples using automated data reduction procedures (see Section IX).

Assay Procedure Flow Chart

Day One						Day Three		Day Four		
Set-up	Step 1	Step 2 & 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9	Step 10	Step 11, 12, & 13
Tube Number	Add Buffer	Add Standard / QC Sample	Add Matrix Solution-HPI	Add Human Proinsulin Antibody	Vortex, Cover, and Incubate 48 hrs at RT	Add I-125 Human Proinsulin Tracer	Vortex, and Incubate 22 hrs. at RT	Add Precipitating Reagent	Vortex, and Incubate 20 min. at 4°C	Centrifuge for 25 min.@ 4°C, Decant, and Count
1,2		-	-	-		100 µl		-		
3,4	400 µl	-	-	-		100 µl		1.0 ml		
5,6	200 µl	-	100 µl	100 µl		100 µl		1.0 ml		
7,8	-	200 µl of 2.0 pM	100 µl	100 µl		100 µl		1.0 ml		
9,10	-	200 µl of 5.0 pM	100 µl	100 µl		100 µl		1.0 ml		
11,12	-	200 µl of 10 pM	100 µl	100 µl		100 µl		1.0 ml		
13,14	-	200 µl of 20 pM	100 µl	100 µl		100 µl		1.0 ml		
15,16	-	200 µl of 50 pM	100 µl	100 µl		100 µl		1.0 ml		
17,18	-	200 µl of 100 pM	100 µl	100 µl		100 µl		1.0 ml		
19,20	-	200 µl of QC 1	100 µl	100 µl		100 µl		1.0 ml		
21,22	-	200 µl of QC 2	100 µl	100 µl		100 µl		1.0 ml		
23,24	100 µl	200 µl of unknown	-	100 µl		100 µl		1.0 ml		
25-n	100 µl	200 µl of unknown	-	100 µl		100 µl		1.0 ml		

IX. CALCULATIONS

A. Explanation

The calculations for Human Proinsulin can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package. Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data.

NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction.

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (Reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.

2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.

3. Calculate the percentage of tracer bound
   (Total Binding Counts/Total Counts) X 100
   This should be 35-50%.

4. Calculate the percentage of total binding (%B/Bo) for standard and sample

   %B/Bo = (Sample or Standard/Total Binding)X100

5. Plot the % B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.

6. Construct the reference curve by joining the points with a smooth curve.

7. Determine the pM of Human Proinsulin in the unknown samples and controls by interpolation of the reference curve.

   NOTE: When sample volumes assayed differ from 200 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 100 µl of sample is used, then calculated data must be multiplied by 2).

X. INTERPRETATION

A. Acceptance Criteria

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 for the Human Proinsulin assay is 2 pM (200 µl sample size).

4. The limit of linearity for the Human Proinsulin assay is 100 pM (200 µl sample size). Any result greater than 100 pM should be repeated on dilution using Assay Buffer as a diluent.

XI. NORMAL FASTING RANGE

7.9 ± 1.5 pM

Linco recommends that each laboratory establish a normal fasting range.

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Proinsulin that can be detected by this assay is 2 pM when using a 200 µl sample size.

B. Performance

The following parameters of assay performance are expressed as Mean ± Standard Deviation.

ED_{80} = 10 ± 1 pM
ED_{50} = 23 ± 2 pM
ED_{20} = 52 ± 3 pM

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

Intact Human Proinsulin	100%
Des 31, 32 HPI	95%
Des 64, 65 HPI	<0.1%
Human Insulin	<0.1%
Human C-Peptide	<0.1%
Bovine Proinsulin	ND
Porcine Proinsulin	ND
Glucagon	ND
Human IGF-I	ND
Human IGF-II	ND
Somatostatin	ND
Pancreatic Polypeptide	ND

ND-not detectable

D. Precision

Within and Between Assay Variation

Sample No.	Mean pM	Within % CV	Between % CV
1	4.7	6.9	7.7
2	7.5	5.0	10.1
3	17.8	2.0	5.0
4	40.8	1.5	1.5

Within and between assay variation was performed on four human serum samples containing varying concentrations of Human Proinsulin. Data (mean and % CV) shown are from five duplicate determinations of each serum sample in four separate assays.

XII. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike & Recovery of Proinsulin in Human Serum

Sample No.	Proinsulin Added pM	Observed pM	Expected pM	% Recovery
1	0	4.0	-	-
2	5	8.3	9.0	92
3	10	12.8	14.0	91
4	20	22.3	24.0	93

Varying concentrations of Human Proinsulin were added to four human serum samples and the Proinsulin content was determined by RIA. Mean of the observed levels from four duplicate determinations in four separate assays are shown. Percent recovery was calculated on the observed vs. expected.

F. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled	Observed pM	Expected pM	% Of Expected
1	200 µl	40.8	40.8	100
	150 µl	43.2		106
	100 µl	44.9		110
2	200 µl	17.8	17.8	100
	150 µl	19.3		108
	100 µl	21.3		120
3	200 µl	14.3	14.3	100
	150 µl	15.5		108
	100 µl	16.2		113
4	200 µl	12.6	12.6	100
	150 µl	13.0		103
	100 µl	14.4		114

Aliquots of pooled human serum containing varying concentrations of Proinsulin were analyzed in the volumes indicated. Dilution factors of 1, 1.33, and 2 representing 200 µl, 150 µl, and 100 µl, respectively, were applied in calculating observed concentrations. Mean Proinsulin levels and percent of expected for four separate assays are shown.

XII. ASSAY CHARACTERISTICS (continued)

G. Example of Assay Results

This data is presented as an example only and should not be used in lieu of a standard curve prepared with each assay.

Tube #	ID	CPM	Ave CPM	Ave Net CPM	% B/Bo	pM
1	Totals	16329				
2	″	16134	16232			
3	NSB	585				
4	″	555	570			
5	Bo	6963				
6	″	7096	7030	6460		
Standards						
7	2 pM	6797				
8		6625	6711	6141	95.1	
9	5 pM	6114				
10		6395	6255	5685	88.0	
11	10 pM	5624				
12		5574	5599	5029	77.9	
13	20 pM	4050				
14		4152	4101	3531	54.7	
15	50 pM	1895				
16		1883	1889	1319	20.4	
17	100 pM	1219				
18		1144	1182	612	9.5	
Controls/Unknown						
19	QC 1	5795				
20		5906	5851	5281	81.7	8.16
21	QC 2	2905				
22		2931	2918	2348	36.3	31.6

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control (QC) specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for Quality Control decisions and is also responsible for making this system a written part of their laboratory manual. 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.

Recommended batch analysis decision using two controls (Westgard Rules):[3]

1. When both controls are within ±2 SD. Decision: Approve batch and release analyte results.

2. When one control is outside ±2 SD and the second control is within ±2 SD.
   Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.

   Technician check of systems:
   1. Check for calculation errors
   2. Repeat standards and controls
   3. Check reagent solutions
   4. Check instrument

XIV. REPLACEMENT REAGENTS

Reagent	**Cat. #**
^{125}I-Human Proinsulin (<3 uCi, 111 kBq)	9015
Human Proinsulin Label Hydrating Buffer (27ml)	LHB-15
Human Proinsulin Standards (2 ml each)	8015-K
Matrix Solution (6 ml)	GP-0015
Human Proinsulin Antibody (26 ml)	1015-K
Precipitating Reagent (260 ml)	PR-15
QC 1&2 (1 ml each)	6000-K
Assay Buffer (40 ml)	AB-P

XV. 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

NOTE: Appropriate license from NRC (or equivalent) must be on file at LINCO before radioactive orders can be shipped.

TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400
FAX ORDERS: (636) 441-8050
MAIL ORDERS: LINCO Research
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

XV. ORDERING INFORMATION (continued)

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 humans 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.

XVI. REFERENCES

1. Feldman, H. and Rodbard, D. "Mathematical Theory of Radioimmunoassay", in: W.D. Odell and Doughaday, W.H. (Ed.), Principles of Competitive Protein-Binding Assays. Philadelphia: J.B. Leppincott Company; pp 158-203, 1971.

2. Thorell, J.I. *Scand. J. Clin. Lab. Invest*. 31:187, 1973.

3. Westgard, J.O., et. al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem*. 27:493-501, 1981.