Quantikine® ELISA

Human Proinsulin Immunoassay

Catalog Number DPINS0

For the quantitative determination of human Proinsulin concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Insulin is a peptide hormone that is critical for glucose homeostasis. The mature Insulin peptide is derived from Proinsulin, which includes the Insulin A and B chains connected by a peptide fragment (Insulin C-peptide). Proinsulin is processed within the endoplasmic reticulum of pancreatic β cells into equimolar ratios of mature Insulin and Insulin C-peptide. Proinsulin, a single chain peptide member of the Insulin family of proteins, is synthesized as a 110 amino acid (aa) prepropeptide that includes a 24 aa signal sequence. Human Proinsulin shares 82% aa sequence identity with the mouse and rat protein and 80%, 88%, 86%, 81%, and 88% aa sequence identity with bovine, equine, porcine, feline, and canine Proinsulin, respectively. While mature Insulin is critical for glucose homeostasis, and Insulin C-Peptide facilitates microvascular circulation, nerve conduction, and renal function, Proinsulin has low metabolic activity. However, Proinsulin is biologically active and may act to regulate embryonic morphogenic development (1).

Proinsulin has low binding affinity for the Insulin Receptor B isoform (IR-B) and the Insulin-like Growth Factor I Receptor (IGF R1) (2-4). However, it binds the Insulin Receptor A isoform (IR-A) with high affinity resulting in cell proliferation and migration in cells expressing IR-A, suggesting that Proinsulin may induce biological effects through IR-A activation (4).

Circulating levels of Proinsulin are altered during dysfunctional glucose regulation and increase with age (5). Impaired processing of Proinsulin to mature Insulin is an early abnormality of pancreatic β cell dysfunction that results in elevated Proinsulin:Insulin ratios (6, 7). Serum Proinsulin levels increase with age and are elevated in patients with metabolic syndrome and Type II Diabetes Mellitus (7-11). Proinsulin levels and the Proinsulin:Insulin ratio are also elevated in response to pancreatic lipotoxicity, which is characterized by excess lipid deposition in the pancreas, as lipotoxicity inhibits the processing of Proinsulin to mature Insulin (12).

The Quantikine Human Proinsulin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Proinsulin in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human Proinsulin and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Proinsulin showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human Proinsulin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Proinsulin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Proinsulin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Proinsulin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Proinsulin bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Proinsulin Microplate	894662	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Proinsulin.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Proinsulin Conjugate	894663	21 mL of a monoclonal antibody specific for human Proinsulin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Proinsulin Standard	894664	Recombinant human Proinsulin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-66	896010	21 mL of a buffered protein base with preservatives. *Used diluted 1:5 for cell culture supernate samples. Used undiluted for serum/plasma samples.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Human Proinsulin Controls (optional; available from R&D Systems).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Hemolyzed samples are not suitable for use in this assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

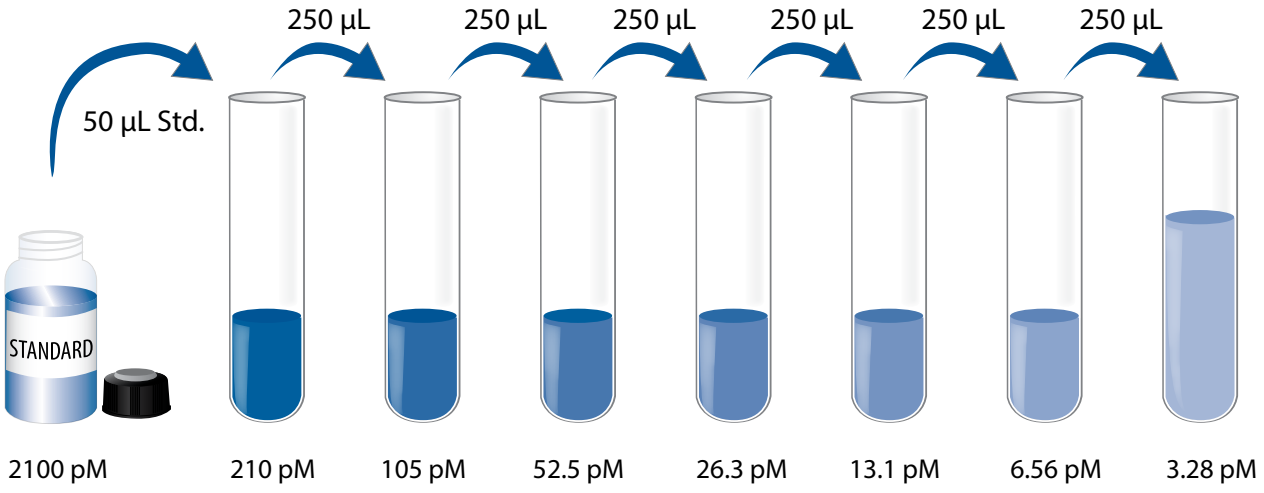
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-66 (diluted 1:5) - **For cell culture supernate samples only.** Add 20 mL of Calibrator Diluent RD5-66 to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-66 (diluted 1:5).

Human Proinsulin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Proinsulin Standard with deionized or distilled water. This reconstitution produces a stock solution of 2100 pM. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-66 (diluted 1:5) (*for cell culture supernate samples*) or Calibrator Diluent RD5-66 (*for serum/plasma samples*) into the 210 pM tube. Pipette 250 µL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 210 pM standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pM).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1W to each well.
4. Add 100 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human Proinsulin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

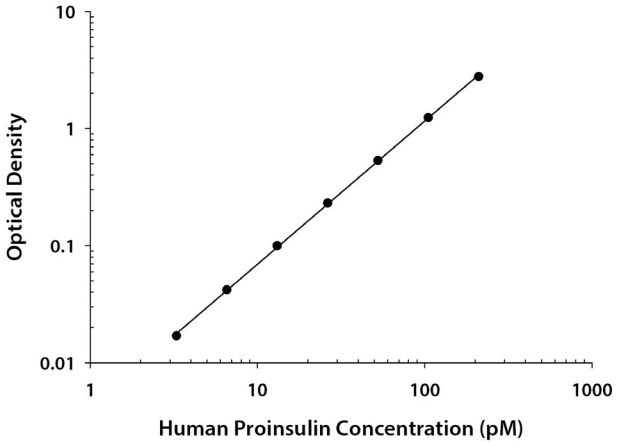
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Proinsulin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

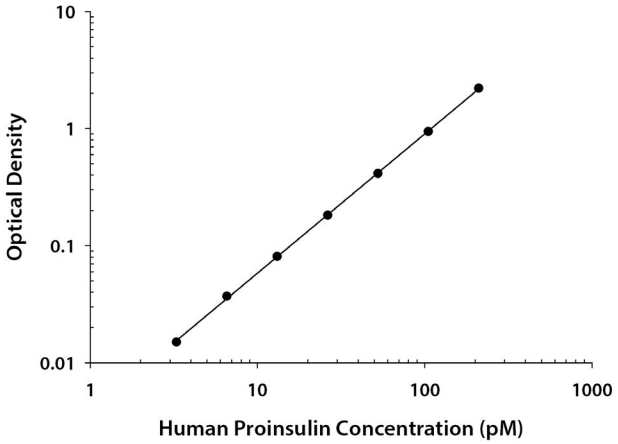
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pM)	O.D.	Average	Corrected
0	0.011 0.011	0.011	—
3.28	0.027 0.028	0.028	0.017
6.56	0.053 0.053	0.053	0.042
13.1	0.109 0.112	0.111	0.100
26.3	0.239 0.245	0.242	0.231
52.5	0.541 0.549	0.545	0.534
105	1.249 1.253	1.251	1.240
210	2.773 2.807	2.790	2.779

SERUM/PLASMA ASSAY



(pM)	O.D.	Average	Corrected
0	0.013 0.013	0.013	—
3.28	0.028 0.028	0.028	0.015
6.56	0.049 0.051	0.050	0.037
13.1	0.093 0.094	0.094	0.081
26.3	0.193 0.196	0.195	0.182
52.5	0.424 0.427	0.426	0.413
105	0.939 0.974	0.957	0.944
210	2.216 2.225	2.221	2.208

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pM)	15.5	48.8	96.2	14.8	46.0	91.2
Standard deviation	0.304	0.643	0.956	1.31	2.75	5.28
CV (%)	2.0	1.3	1.0	8.9	5.8	6.9

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pM)	21.1	62.7	119	18.0	55.1	109
Standard deviation	0.440	0.745	1.40	1.90	3.97	6.91
CV (%)	2.1	1.2	1.2	10.6	7.2	6.3

RECOVERY

The recovery of human Proinsulin spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	104	94-119%
Serum (n=4)	95	80-105%
EDTA plasma (n=4)	92	86-108%
Heparin plasma (n=4)	94	90-100%

SENSITIVITY

Fifty-two assays were evaluated and the minimum detectable dose (MDD) of human Proinsulin ranged from 0.165-1.43 pM. The mean MDD was 0.525 pM.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human Proinsulin were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	101	99	97	99
	Range (%)	93-114	97-102	93-104	89-105
1:4	Average % of Expected	96	103	102	102
	Range (%)	87-102	97-108	95-109	96-108
1:8	Average % of Expected	99	105	108	106
	Range (%)	87-107	99-110	102-117	100-110
1:16	Average % of Expected	96	103	104	103
	Range (%)	82-102	99-105	97-111	96-109

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human Proinsulin manufactured at R&D Systems.

The NIBSC/WHO Proinsulin International Reference Reagent 84/611 was evaluated in this kit. The dose response curve of the reference reagent 84/611 parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine Human Proinsulin kit to approximate NIBSC/WHO 84/611 pM, use the equation below.

NIBSC/WHO (84/611) approximate value (pM) = 1.262 x Quantikine Human Proinsulin value (pM)

Note: *Based on data generated in February 2014.*

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Proinsulin in this assay. No medical histories were available for the donors used in this study.

	Mean of Detectable (pM)	% Detectable	Range (pM)
Serum (n=35)	7.76	51	ND-23.0
EDTA plasma (n=35)	8.05	51	ND-24.0
Heparin plasma (n=35)	8.12	51	ND-22.8

ND=Non-detectable

Cell Culture Supernates - Seventy-nine cell lines were tested for human Proinsulin. No detectable levels were observed.

SPECIFICITY

This assay recognizes natural and recombinant human Proinsulin.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Proinsulin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
GLP-1
IGF-I
IGF-II
INSL3
Insulin C-Peptide
Relaxin-1
Relaxin-2
Relaxin-3

Recombinant mouse:
IGF-I
IGF-II

Recombinant rat:
IGF-I

Recombinant human Insulin interferes at concentrations > 3450 pM.

REFERENCES

1. Hernandez-Sanchez, C. *et al.* (2006) Diabetologia **49**:1142.
2. Burguera, B. *et al.* (1991) J. Clin. Endocrinol. Metab. **72**:1238.
3. Podlecki, D.A. *et al.* (1984) Diabetes **33**:111.
4. Malaguarnera, R. *et al.* (2012) Endocrinology **153**:2152.
5. Davies, M.J. *et al.* (1993) Diabet. Med. **10**:313.
6. Bergman, R.N. *et al.* (2002) Eur. J. Clin. Invest. **32 Suppl 3**:35.
7. Kahn, S.E. and P.A. Halban (1997) Diabetes **46**:1725.
8. Bryhni, B. *et al.* (2010) BMC Endocr. Disord. **10**:21.
9. Pradhan, A.D. *et al.* (2003) Am. J. Med. **114**:438.
10. Grill, V. *et al.* (2002) Am. J. Epidemiol. **155**:834.
11. Mykkanen, L. *et al.* (1995) Diabetologia **38**:1176.
12. Bjorklund, A. and V. Grill (1999) Diabetes **48**:1409.