

IMMUNOENZYMOMETRIC ASSAY FOR THE QUANTATIVE DETERMINATION OF PROINSULIN IN HUMAN SERUM.

E-BX-96 - 96 Assays

FOR IN VITRO DIAGNOSTIC USE ONLY

CLINICAL APPLICATIONS

Proinsulin (PI), a polypeptide of 9390 MW (86 amino acids), is synthesized in the β cells of the Islets of Langerhans in the pancreas(1). The sequence of proinsulin is highly conserved in mammalian species and is homologous with IGF-1 and IGF-2 (2). This protein is processed to C-peptide and insulin forms (3). Both are secreted in equimolar amounts into the blood. In normal individuals, proinsulin is present in the circulation in very low concentrations (typical basal values 26 pmol/L). The level of proinsulin in serum can be a reflection of β cell status and a consequence of dysfunction of PI processing and/or secretion (4). Proinsulin like material is increased in clinical conditions as insulinoma, familial hyperinsulinemia, non-insulin dependent diabetes mellitus (5,6,7,8).

PRINCIPLE OF THE ASSAY (I)

Proinsulin kit is a sensitive two-site sandwich enzyme linked immunosorbent assay. The microtiter plates are coated with a monoclonal antibody (S2) specific for epitope at the C-peptide/insuline A chain junction. S2 is able to bind intact PI, des (31,32)-PI and split (32,33)-PI but not insulin, C-peptide and the other "des" and "split" forms.

An aliquot of patient sample is added to the wells and incubated. The wells are then washed to remove unbound antibody and other serum compounds. In a second incubation time, an enzyme labelled anti-proinsulin antibody (S53) is incubated in the wells. This antibody is specific for the epitopes at insulin ? chain/C-peptide junction. S53 is able to bind to intact PI, des (64,65)-PI and split (65,66)-PI but not insulin, C-peptide and other "des" and "split" forms. The combination of the two Mabs has the ability to detect only the intact human proinsulin.

After washing away any unbound enzyme labelled anti-proinsulin antibody, the enzyme activity is measured by adding a chromogen substrate. The intensity of colour development is proportional to the concentration of proinsulin in the patient sample.

(I) Paule Housa et al. "First direct assay for intact human proinsulin". Clinical Chemistry 44:7, 15414-1519 (1998)

REAGENTS PROVIDED WITH THE KIT

- Reagents are sufficient for 96 wells.
 - Store the kit at 2-8°C.
 - The expiry date of each reagent is shown on the vial label.
- 1. Coated microtiterplate** : 96 breakable wells coated with a proinsulin specific antibody . Keep unused wells at 2-8°C, protected from moisture in the provided aluminium bag and carefully sealed. Store at 2 – 8°C
 - 2. Proinsulin 0 calibrator** : 1 vial of lyophilised calibrator. *Reconstitute with 3 ml of distilled water.* Blue coded. After reconstitution, keep the calibrator at –20°C (freeze-defreeze cycle: max 2 times). Preservatives : Merthiolate. Store lyophilized at 2 – 8°C
 - 3. Proinsulin 1 - 5 calibrators:** 5 vials of lyophilised calibrator. *Reconstitute with 1 ml of distilled water.* Blue coded. After reconstitution, keep the calibrator at –20°C (freeze-defreeze cycle: max 2 times). For the exact value, refer to the data sheet included. Preservatives : Merthiolate. Store lyophilized at 2 – 8°C

- 4. HRP CONJUGATE** : 1 vial (11 ml) of anti-human proinsulin conjugated to horseradish peroxidase (HRPO). Preservatives : Neomycin and Merthiolate. Store at 2 – 8°C.
- 5. Control serum** : 2 vials of lyophilised sample. Reconstitute with 1 ml of distilled water. Blue coded. After reconstitution, keep the control serum at –20°C (freeze-defreeze cycle: max 2 times) For the exact value, refer to the data sheet included. Preservatives : Merthiolate. Store lyophilised at 2 – 8°C.
- 6. Washing solution** : 1 vial (40 ml) of buffer with Tween 20. Preservative: Sulfate Streptomycin and Amphotericin. Bring the vial content to 400 ml (final volume) with distilled water. The diluted washing solution is stable for 6 months at 2 - 8°C. Store at 2 – 8°C
- 7. Chromogen** : 1 vial (15ml) of Tetramethylbenzidine in citrate-phosphate buffer and DMSO. Ready for use. Store at 2 – 8°C
- 8. Substrate buffer** : 1 vial (15ml) of citrate-phosphate buffer containing H_2O_2 . Ready for use. Note: **before use, make a 1+1 dilution with equal volumes of chromogen and substrate buffer in glassware.** Avoid direct light exposure and use within 1 hour from preparation. Store at 2 – 8°C
- 9. Blocking reagent** : 1 vial (15ml) of 1N H_2SO_4 . Ready for use. Store at 2 – 8°C

KIT REAGENTS

Reagents	Quantity	Physical state
Wells	96	Ready for use
Calibrator 0	1 x 3 ml	Lyophilised
Calibrators 1 – 5	5 x 1 ml	Lyophilised
Control Sera	2 x 1 ml	Lyophilised
HRP conjugate	1 x 11 ml	Ready for use
Washing Solution	1 x 40 ml	Concentrated 10 x
Chromogen	1 x 15 ml	Ready for use
Substrate Buffer	1 x 15 ml	Ready for use
Blocking Reagent	1 x 15 ml	Ready for use

MATERIAL REQUIRED BUT NOT SUPPLIED

- Adjustable, automatic micropipettes with disposable tips.
- Graduated cylinder.
- Aspiration pump or automated well washing device.
- Microtiterplate spectrophotometer capable of measuring absorbances within a 0-3.0 A interval at 450 and 405 nm.
- Millimetric graph paper.
- Orbital shaker adjustable at 150 rpm.
- Distilled H_2O .

WARNINGS AND PRECAUTIONS

In order to obtain reproducible results, the following rules must be observed:

- Do not mix reagents of different lots.
- Do not use reagents beyond their expiry date.
- Use thoroughly clean glassware.
- Use distilled water, stored in clean containers.
- Avoid any contamination among samples; for this purpose, disposable tips should be used for each sample and reagent.

In order to avoid personal and environmental contamination, the following precautions must be observed:

- Use disposable gloves while handling potentially infectious material and performing the assay.
 - Do not pipette reagents by mouth.
 - Do not smoke, eat, drink or apply cosmetics during the assay.
 - All material of human origin used for the preparation of this kit tested negative for HBsAg, anti-HIV and anti-HCV. Since no test at present can guarantee complete absence of these viruses, all samples and reagents used for the assay must be considered potentially infectious. Therefore, the assay waste must be decontaminated and disposed of, in accordance with established safety procedures.
 - Disposable ignitable material must be incinerated; disposable non-ignitable material must be sterilized in autoclave for at least 1 hour at 121°C. Liquid wastes must be added with sodium hypochlorite at a final concentration of 3%. Let the hypochlorite act for at least 30 minutes. Liquid wastes containing acid must be neutralized with appropriate amounts of base, before treating with sodium hypochlorite.
 - Chromogen and Blocking Reagent should be handled with care. Avoid contact with skin, eyes and mucous membranes. In case of accident rinse thoroughly with running water.
 - Avoid splashing and aerosol formation; in case of spilling, wash carefully with a 3% sodium hypochlorite solution and dispose of this cleaning liquid as potentially infectious waste.
- Some reagents contain sodium azide as preservative; to prevent build-up of explosive metal azides in lead and copper plumbing, reagents should be discarded by flushing the drain with large amounts of water.

SPECIMEN COLLECTION AND PREPARATION

It is recommended to use serum. Avoid using hemolized, lipemic or bacterially contaminated sera. Thoroughly mix thawed specimens before assay and avoid repeated freeze/thawing cycles which may cause loss of antibody activity and give erroneous results. Samples may be stored at 2°- 8°C for up to 24 hours. For long term storage, samples should be stored frozen at -20°C or lower.

ASSAY SCHEME

Wells	Blank	Calibrator (0-5)	Control Sera	Samples
Reagents				
Calibrator (0 - 5)	----	100 µl	----	----
Control Sera	----	----	100 µl	----
Samples	----	----	----	100 µl
- Incubate: 30' at R.T., orbital shaker (150 rpm)				
- Aspirate and wash: 3 X 300 µl.				
Conjugate	-----	100 µl	100 µl	100 µl
- Incubate: 60' at R.T., orbital shaker (150 rpm)				
- Aspirate and wash: 3 x 300 µl				
Chrom-Substrate	200 µl	200 µl	200 µl	200 µl
- Incubate: 15' at R.T., orbital shaker (150 rpm)				
Blocking Reagent	100 µl	100 µl	100 µl	100 µl
- Read : 450-405 nm and 620 nm as reference.				

ASSAY PROCEDURE

- Allow reagents and samples to warm up at room temperature.
 - Mix samples by inversion before use.
 - The samples are not diluted.
1. Allocate the wells of microtiterplate for calibrators, control sera and samples
 2. Pipette 100µl of each calibrators, control serum and sample into the corresponding wells.

3. Incubate for 30 minutes at room temperature on an orbital shaker (150rpm) (NB. The incubation time begins after the last sample addition)
4. Wash the wells 3 times with 300µl of diluted washing solution. Aspirate all liquid from the wells.
5. Add 100µl of HRP conjugate into the wells.
6. Incubate for 60 minutes at room temperature on an orbital shaker (150rpm).
7. Wash the wells 3 times with 300µl of diluted washing solution. Aspirate all liquid from the wells.
8. Pipette 200µl of substrate-chromogen solution into the wells
9. Incubate 15 minutes at room temperature on an orbital shaker (150rpm), avoid direct light exposure (NB. The incubation time begins after the first TMB addition)
10. Pipette 100µl of blocking reagent into the wells
11. Read the absorbance of the wells (450, 405 and 620nm (as reference)). Reading must be completed within 20 minutes from the end of the assay.

CALCULATION OF RESULTS

In order to obtain a better sensitivity, the present method employs spectrophotometric reading at two wavelengths (450 and 405 nm). For all O.D. overflow at 450nm, multiplied the O.D. 405nm by the correction factor calculated by the ratio between O.D. 450nm and O.D. 405nm. To establish this factor, used a sample reading at 405nm and 450nm.

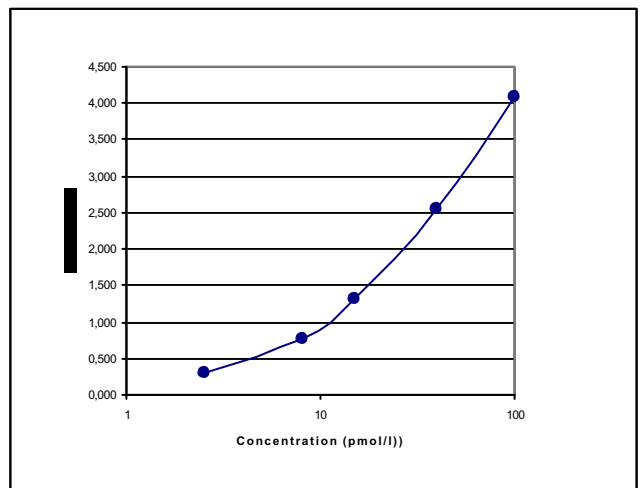
Draw a calibration curve on millimetric graph paper, by plotting the calibrators concentration (x-axis) against the absorbance obtained for each standard (y-axis, logit). Corresponding Proinsulin concentrations in pmol/l are obtained by interpolating the absorbances of each sample on the calibration curve.

EXAMPLE OF CALCULATION

The values shown below must be considered as an example and must not be used in place of experimental data.

Description	Concentration (pmol/l)	O.D	B/Bmax (%)
Calibrator 0	0	0,065	1,6
Calibrator 1	2.5	0,305	7,4
Calibrator 2	8	0,780	19
Calibrator 3	15	1,304	32
Calibrator 4	40	2,556	62
Calibrator 5	100	4,094	100
Control serum 1	9.8	0.924	
P1	1.6	0.217	
P2	6.9	0.690	
P3	14.7	1.279	
P4	31.6	2.208	

TYPICAL CALIBRATION CURVE



NORMAL VALUES

The normal values determined are only indicative since they may be affected by various agents. We recommend that each laboratory establish its own normal range.

	n	Mean & SD (pmol/l)
Non obese fasting patients	16	2.56 & 1.28
Fasting IDDM	9	4.67 & 3.85
Non-fasting IDDM	13	16.25 & 17.75

PERFORMANCES OF THE ASSAY

SENSITIVITY

Analytical sensitivity

The sensitivity was calculated based upon the calibration curve and expressed as the minimal dose showing a significant difference from the Zero Calibrator (mean value + 2 S.D.). This dose is 0.50 pmol/l.

PRECISION

Intra-assay

Serum	Mean	±	S.D.	C.V. (%)	Replicates no.
a	7.1	±	0.5	6.6	22
b	14.9	±	0.3	2.0	22
c	31.1	±	1.3	4.3	22

Inter-assay

Serum	Mean	±	S.D.	C.V. (%)	Replicates no.
a	6.1	±	0.1	2.4	9
b	14.3	±	0.3	1.8	9
c	29.2	±	0.7	2.5	9

ACCURACY

Recovery Test

Samples (p/mol/l)	Measured (p/mol/l)	Expected (p/mol/l)	Recovery (%)
S1	3,6		
S1 + Cal 0	2,0	1,8	110,3
S1 + Cal 1	2,9	3,0	95,6
S1 + Cal 2	5,3	5,8	91,1
S1 + Cal 3	8,2	9,3	88,0
S1 + Cal 4	18,2	21,8	83,4
S1 + Cal 5	46,8	51,8	90,4

Parallelism Test

Dilution	Expected (pmol/l)	Measured (pmol/l)	Recovery (%)
1/1	29,7	-	100,0
1/2	16,5	14,8	111,3
1/4	8,0	7,4	108,4
1/8	4,2	3,7	113,3
1/10	3,3	3,0	110,2
1/20	1,5	1,5	101,2

SPECIFICITY

Cross-reactivity

The following peptides were tested and no cross-reactivity has been observed :

Human Insulin , < 10 000 pmol/l
Human C-Peptide , 50 000 pmol/L
Des (31,32)- Proinsulin , < 200 pmol/L
Split (32,33)- Proinsulin , 5000 pmol/L
Des (64,65)- Proinsulin , 200 pmol/L
Split (65,66)- Proinsulin , 1000 pmol/L

BIBLIOGRAPHIE - BIBLIOGRAPHY

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