Product Manual

Proinsulin ELISA Kit

Catalog #: ENZ-KIT149-0001

96-Well Kit

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Product Manual

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Please read entire booklet before proceeding with the assay.

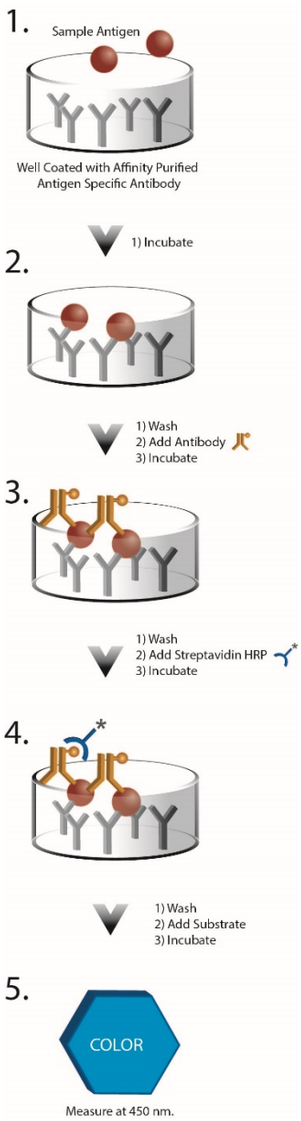
INTRODUCTION

The Proinsulin ELISA kit is a complete immunometric kit for the quantitative determination of proinsulin in human serum and plasma. Please read the complete kit insert before performing this assay.

Type 2 diabetes mellitus is the most common serious metabolic condition in the world, and results from a subnormal response of tissues to insulin (insulin resistance) and a failure of the insulin-secreting beta cells to compensate[1]. Insulin is required for glucose homeostasis and an important precursor protein to insulin is proinsulin. Proinsulin is composed of the A and B subunits of insulin which is connected by the C-peptide region[2]. This 10.5 kDa protein, comprised of 110 amino acids, is synthesized as a single chain that contains a 24 amino acid signal sequence and an 86 amino acid proinsulin propeptide. Proinsulin is processed in the endoplasmic reticulum of pancreatic β-cells and is converted within immature secretory granules into insulin and C-peptide[3]. While mature insulin is required for glucose homeostasis and C-peptide is associated with renal function, proinsulin has low metabolic activity. However, it is biologically active and is a regulator of development in pre-pancreatic embryonic stages[4].

The release of proinsulin is significantly increased in patients with type 2 diabetes as well as in individuals with impaired glucose tolerance (IGT)[5]. This increase in diabetic patients is commonly referred to as hyperproinsulinaemia and could result from a defect in the processing of proinsulin to mature insulin. This defect is an early sign of an abnormality of pancreatic β cell dysfunction and results in elevated proinsulin:insulin ratios[6]. The levels of proinsulin have also been found to increase with age.

PRINCIPLE



1. The kit uses a monoclonal antibody specific to the proinsulin C-peptide immobilized on a microtiter plate. Standards or samples containing proinsulin are added to the plate.
2. After a short incubation the excess sample or standard is washed out and a biotinylated monoclonal antibody to insulin is added. This antibody binds to the Proinsulin captured on the plate.
3. After a short incubation the excess antibody is washed out and Streptavidin conjugated to Horseradish peroxidase (SA-HRP) is added, which binds to the biotin on the bound monoclonal antibody. The plate is then incubated.
4. Once the incubation is complete, excess conjugate is washed out and TMB substrate solution is added and incubated. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of Proinsulin in the sample.

MATERIALS SUPPLIED

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

1. **Proinsulin Microtiter Plate, One Plate of 96 Wells**
   Catalog No. 80-2682

   A plate using break-apart strips coated with a monoclonal antibody specific to the proinsulin C-peptide.

2. **Sample Diluent, 20 mL**
   Catalog No. 80-2706-0020

   Serum-like diluent for standard curve, NSB and sample preparation.

3. **Proinsulin Antibody, Lyophilized**
   Catalog No. 80-2684-0100

   Lyophilized monoclonal antibody specific to insulin.

4. **Antibody Diluent, 14 mL**
   Catalog No. 80-2848

   Buffer for reconstitution and dilution of proinsulin antibody.

5. **Proinsulin Standard, 1 nM**
   Catalog No. 80-2683

   One vial containing 200 µL of 1 nM proinsulin.

6. **Wash Buffer Concentrate, 100 mL**
   Catalog No. 80-1287

   One bottle containing 20x Tris buffered saline containing detergent.

7. **Proinsulin Conjugate, 20 mL**
   Catalog No. 80-2705-0020

   A solution of Streptavidin-conjugated Horseradish Peroxidase.

Protect substrate from prolonged exposure to light.

8. **TMB Substrate, 10 mL**
   Catalog No. 80-0350

   A solution of 3,3’5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide.

Stop solution is caustic. Keep tightly capped.

9. **Stop Solution 2, 10 mL**
   Catalog No. 80-0377

   A 1N solution of hydrochloric acid in water.

10. **Proinsulin Assay Layout Sheet, 1 each**
    Catalog No. 30-0331

11. **Plate Sealer, 3 each**
    Catalog No. 30-0012

This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.

STORAGE

All kit components are stable at 4°C until the kit's expiration date. Shipping conditions may not reflect storage conditions.

***NOTE: After opening, the sample diluent (80-2706) must be stored at or below -20°C and has been shown to tolerate up to three freeze/thaw events with no negative effects to the assay.**

OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater pipet for dispensing 100 µL
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. A microplate shaker
7. Adsorbent paper for blotting
8. Microplate reader capable of reading a 450 nm
9. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.

Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

SAMPLE HANDLING

The Proinsulin ELISA is suitable for measuring proinsulin in human serum and EDTA plasma. Prior to sample analysis in the assay, frozen samples should be slowly thawed at room temperature and vortexed to mix. Samples diluted sufficiently into sample diluent can be read directly from a standard curve. Serum and plasma samples must be diluted at least 1:4 with sample diluent in order to remove matrix interference effects (please refer to the Spike and Recovery section on page 6 for detailed data). The minimum recommended dilution may not be optimal for all samples for the levels of endogenous proinsulin could vary between sample groups. Therefore, it is up to each end user to optimize the dilution for their unique set of samples.

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

SAMPLE MATRIX PROPERTIES

Linearity

Human serum and EDTA plasma samples were diluted 1:4 in sample diluent, spiked with recombinant proinsulin and serially diluted 1:2 in sample diluent. All samples were run in the assay and compared to the standard curve. The results are shown in the table below.

Dilutional Linearity, %		
Dilution	**Serum**	**EDTA Plasma**
1:4	100	100
1:8	108	93
1:16	113	96
1:32	137	113

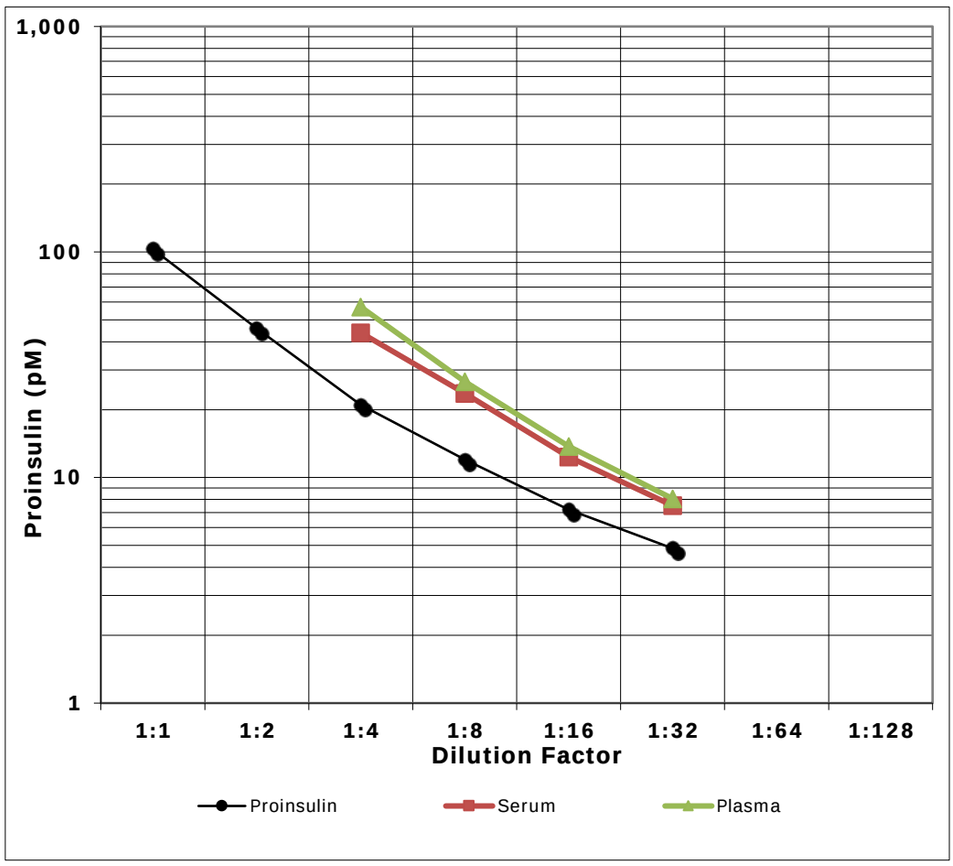
Spike and Recovery

Serum and EDTA plasma samples were diluted 1:4 in sample diluent and then spiked with recombinant proinsulin at a high, medium and low concentration. Matrix background was subtracted and the percent recovery of the spiked proinsulin was determined.

Sample	Spike Concentration, pM	% Recovery	Minimum Recommended Dilution
Serum	45	100.3	1:4
	15	72.3	
	5	111.3	
EDTA Plasma	45	72.9	1:4
	15	74.9	
	5	118.4	

Parallelism

To assess parallelism, human serum and EDTA plasma samples were diluted 1:4 in sample diluent, spiked with recombinant human proinsulin and then serially diluted (1:2) in sample diluent. The samples were then run in the assay. The proinsulin concentration in each sample was assigned using the standard curve. Assigned concentrations were plotted as a function of sample dilution. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted samples of human origin.



Sample handling procedures should be completed prior to reagent preparation.

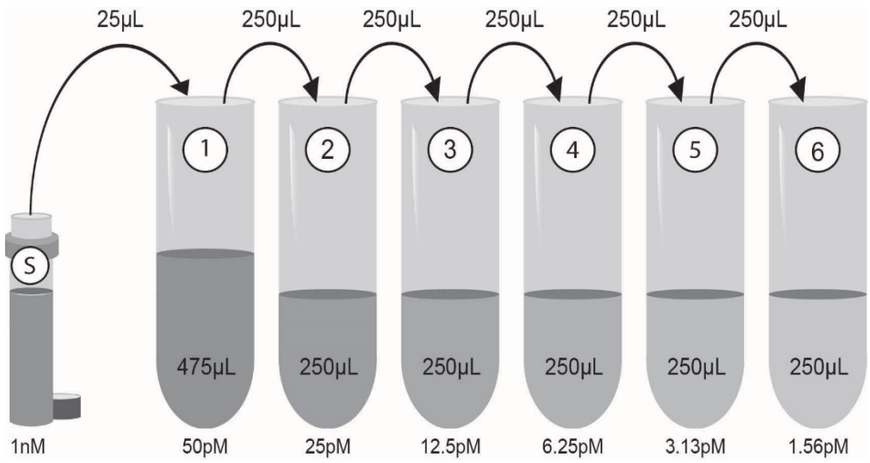
REAGENT PREPARATION

1. **Wash Buffer**

   Prepare Wash buffer by diluting 50 mL of the supplied Wash Buffer concentrate with 950 mL of deionized water. The diluted wash buffer can be stored at room temperature for up to 3 months.

2. **Proinsulin Standard**

   Allow the Proinsulin Standard stock to warm to room temperature. Label six 12x75 mm polypropylene tubes #1 through #6. Add 475 µL of Sample Diluent into tube #1. Add 250 µL of Sample Diluent into tube #2 through tube #6. Add 25 µL of 1 nM Proinsulin Standard stock to tube #1 and vortex gently. Add 250 µL of tube #1 into tube #2 and vortex. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.



   Diluted standards should be used within 60 minutes of preparation. Discard any unused standard dilutions.

3. **Proinsulin Antibody**

   Reconstitute the lyophilized proinsulin antibody in 1.6 mL ofAntibody Diluent to yield a 10x stock. Dilute appropriate volume to 1x (i.e. 0.5 mL of 10x stock into 4.5 mL of Antibody Diluent) and freeze remaining 10x stock at -20ºC. Avoid repeated freeze thaw cycles.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove unneeded wells and return them, with the desiccant, to the plate bag and seal. Store the unused wells at 4°C.

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

Pipet the reagent to the side of the wells to avoid possible contamination.

1. Add 100 µL of Sample Diluent into the S0 (0pM standard) and NSB (Non-specific binding) wells. Leave the Blank and TA (Total Activity) wells empty.
2. Add 100 µL of standards #1 through #6 into the appropriate wells.
3. Add 100 µL of the samples into the appropriate wells.
4. Seal the plate. Incubate at room temperature (RT) on a plate shaker for 1 hour at ~500 rpm*****. **See note.**
5. Empty the contents of the wells and wash by adding a full well volume (~400 µL) of 1X Wash Buffer to each well. Empty or aspirate the wells and repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Add 100 µL of 1X reconstituted Proinsulin Antibody into each well, except the NSB, Blank and TA wells. Add 100 µL Sample Diluent into NSB wells and leave Blank and TA wells empty.
7. Seal the plate. Incubate at RT on a plate shaker for 1 hour at ~500 rpm.
8. Wash as above (Step 5).
9. Add 100 µL of Proinsulin Conjugate to each well, except the Blank and TA wells.
10. Seal the plate and incubate at RT on a plate shaker for 30 minutes at ~500 rpm.
11. Wash as above (Step 5).
12. Dilute Proinsulin Conjugate 1:100 with Sample Diluent and add 5 µL of this dilution to TA wells.
13. Add 100 µL of TMB substrate into all wells.
14. Seal the plate and incubate at RT on a plate shaker for 30 minutes at ~500 rpm.
15. Add 100 µL of Stop Solution into each well.
16. After zeroing the plate reader against the substrate, read optical density at 450 nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*** <u>Note:</u> The plate shaker speed was based on a BellCo Mini Orbital Shaker (mod no. 7744-08096). The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.**

CALCULATION OF RESULTS

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of proinsulin in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program. The concentration of proinsulin can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average NSB OD

2. Using data analysis software, plot the Average Net OD for each standard versus proinsulin concentration in each standard. Samples with concentrations outside of the standard curve range will need to be reanalyzed using alternative dilution(s).

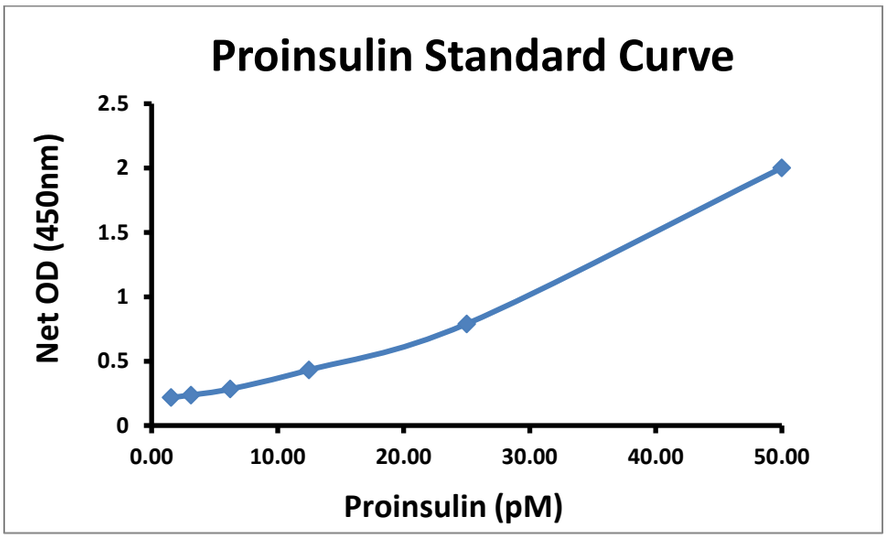
TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results.

Sample	Mean OD	Net OD	Proinsulin (pM)
NSB	0.045	--	--
S0	0.231	0.186	0
S1	2.047	2.002	50
S2	0.835	0.790	25
S3	0.477	0.432	12.5
S4	0.329	0.284	6.25
S5	0.282	0.237	3.13
S6	0.263	0.218	1.56

Typical Standard Curve

A typical standard curve is shown below. This curve must not be used to calculate proinsulin concentrations; each user must run a standard curve for each assay.



PERFORMANCE CHARACTERISTICS

Specificity

The specificity of the assay was determined by running serial dilutions of the analytes, including the cross-reactant, in the assay, fitting the resulting dose response curve(s) to a 4PL curve-fit and determining the ED_{50}. The ED_{50} of the cross-reactant was then divided by the determined ED_{50} of the proinsulin standard curve and multiplied by 100.

Analyte	Cross Reactivity
Proinsulin	100%
Insulin	1.13%

Sensitivity

The sensitivity or limit of detection of the assay is 0.17 pM, determined by interpolation at 2 standard deviations above the background (0pM) of 12 zero standard replicates. Data was used from 10 standard curves.

Intra-assay precision was determined by assaying 20 replicates of three plasma controls containing proinsulin in a single assay.

Intra-assay precision	
pg/mL	**%CV**
26.3	5.5
9.6	6.1
3.7	17.4

Inter-assay precision was determined by measuring plasma controls of varying proinsulin concentrations in multiple assays over several days.

Inter-assay precision	
pg/mL	**%CV**
26.1	6.0
9.0	6.3
3.3	11.6

REFERENCES

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