



Human Proinsulin ELISA Kit

**Catalog Number
EK0468**

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

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

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DETECTION RANGE

156 pg/ml - 10000 pg/ml

SENSITIVITY

The minimum detectable dose was 70pg/mL.

SPECIFICITY

This assay recognizes both natural and recombinant human Proinsulin. The factors listed below were prepared at 200ng/ml in Standard /sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

Table 1: Factors assayed for cross-reactivity

Recombinant human
IGF- I
IGF- II
Insulin

PRECISION

The coefficient of variation of both intra-assay and inter-assay were less than 10%.

MATERIALS PROVIDED

1. Aluminium pouches with a Microwell Plate coated with antibody to human Proinsulin (8X12)
2. 2 vials human Proinsulin Standard lyophilized, 1000 pg/ml upon reconstitution
3. 2 vials concentrated Biotin-Conjugate anti-human Proinsulin antibody
4. 2 vials Streptavidin-HRP solution
5. 4 bottle Standard /sample Diluent
6. 1 bottle Biotin-Conjugate antibody Diluent
7. 1 bottle Streptavidin-HRP Diluent
8. 1 bottle Wash Buffer Concentrate 20x (PBS with 1% Tween-20)

9. 1 vial Substrate Solution
10. 1 vial Stop Solution
11. 4 pieces Adhesive Films
12. package insert

STORAGE

Table 2: Storage of the kit

Unopened Kit	Store at 2 – 8°C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Standard /sample Diluent	May be stored for up to 1 month at 2 – 8°C.**
	Concentrated Biotin-Conjugate	
	Streptavidin-HRP solution	
	Biotin-Conjugate antibody Diluent	
	Streptavidin-HRP Diluent	
	Wash Buffer Concentrate 20x	
	Substrate Solution	
	Stop Solution	
	Standard	Aliquot and store for up to 1 month at -20°C. Avoid repeated freeze-thaw cycles. Diluted standard shall not be reused.
Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 – 8°C.**	

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

THE REQUIRED ITEMS (not provided, but can help to buy):

1. Microplate reader (450nm).
2. Micro-pipette and tips: 0.5-10, 2-20, 20-200, 200-1000µL.

3. 37 °C incubator, double-distilled water or deionized water, coordinate paper, graduated cylinder.

PRECAUTIONS FOR USE

1. Store kit reagents between 2°C and 8°C. After use all reagents should be immediately returned to cold storage (2°C to 8°C).
2. Please perform simple centrifugation to collect the liquid before use.
3. To avoid cross contamination, please use disposable pipette tips.
4. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material. Avoid contact of skin or mucous membranes with kit reagents or specimens. In the case of contact with skin or eyes wash immediately with water.
5. Use clean, dedicated reagent trays for dispensing the washing liquid, conjugate and substrate reagent. Mix all reagents and samples well before use.
6. After washing microtiter plate should be fully pat dried. Do not use absorbent paper directly into the enzyme reaction wells.
7. Do not mix or substitute reagents with those from other lots or other sources. Do not use kit reagents beyond expiration date on label.
8. Each sample, standard, blank and optional control samples should be assayed in duplicate or triplicate.
9. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency or Shake by hand at 10min interval when there is no vortexer.
10. Avoid microtiter plates drying during the operation.
11. Dilute samples at the appropriate multiple, and make the sample values fall within the standard curve. If samples generate values higher than the highest standard, dilute the samples and repeat the assay.
12. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time and temperature, and kit age can cause variation in binding.
13. This method can effectively eliminate the interference of the soluble receptors, binding proteins and other factors in biological samples.

SAMPLE COLLECTION AND STORAGE

1. **Cell Culture Supernates** - Remove particulates by centrifugation.
2. **Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum, avoid hemolysis and high blood lipid samples.
3. **Plasma** - Recommended EDTA as an anticoagulant in plasma. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection.
4. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freeze-thaw cycles.
5. Dilute samples at the appropriate multiple (recommended to do pre-test to determine the dilution factor).

REAGENT PREPARATION

1. Bring all reagents to room temperature before use.
2. **Wash Buffer** - Dilute 10mL of Wash Buffer Concentrate into deionized or distilled water to prepare 200mL of Wash Buffer. If crystals have formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. **Standard** - Reconstitute the Standard with 1.0mL of Standard /sample Diluent. This reconstitution produces a stock solution of 10000 pg /mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 500µL of Standard/sample Diluent into the 5000 pg/mL tube and the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 10000 pg/mL standard serves as the high standard. The Standard/ sample Diluent serves as the zero standard (0 pg/mL).

**If you do not run out of re-melting standard, store it at -20°C.
Diluted standard shall not be reused.**

4. Working solution of Biotin-Conjugate anti-human Proinsulin antibody: Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

The working solution should be used within one day after dilution.

5. Working solution of Streptavidin-HRP: Make a 1:100 dilution of the

concentrated Streptavidin-HRP solution with the Streptavidin-HRP Diluent in a clean plastic tube.

The working solution should be used within one day after dilution.

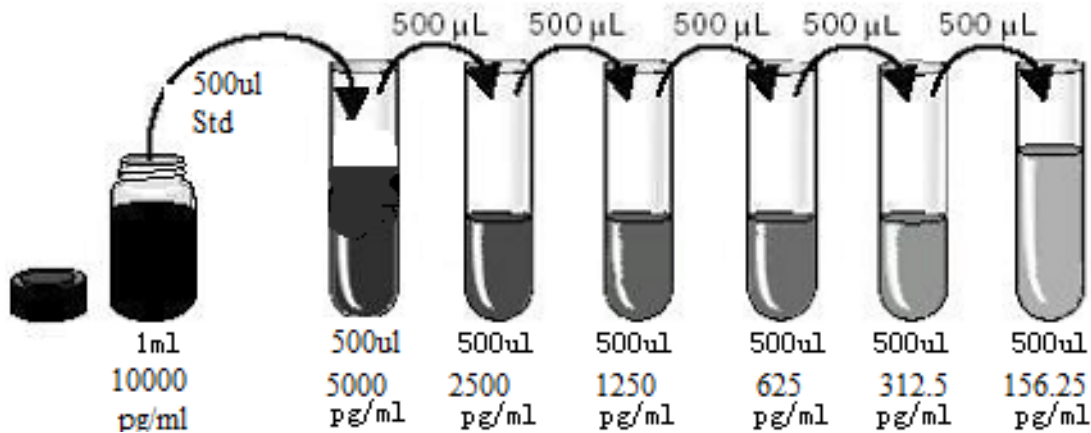


Figure 1: Preparation of Proinsulin standard dilutions

GENERAL ELISA PROTOCOL

1. Prepare all reagents and working standards as directed in the previous sections.
2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2-8°C sealed tightly.
3. Add 100µL of Standard, control, or sample, per well, then add 50µL of the working solution of Biotin-Conjugate to each well. Cover with the adhesive strip provided and incubate 2 hours at RT. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency.
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (350µL) using a squirt bottle, manifold dispenser or auto-washer. 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.

5. Add 100 μ L of the working solution of Streptavidin-HRP to each well. Cover with a new adhesive strip and incubate for 30 minutes at RT. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 μ L of Substrate Solution to each well. Incubate for 10-20 minutes at RT. Avoid placing the plate in direct light.
8. Add 100 μ L of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.(optionally 650nm as the reference wave length;610-650nm is acceptable)

ASSAY PROCEDURE SUMMARY

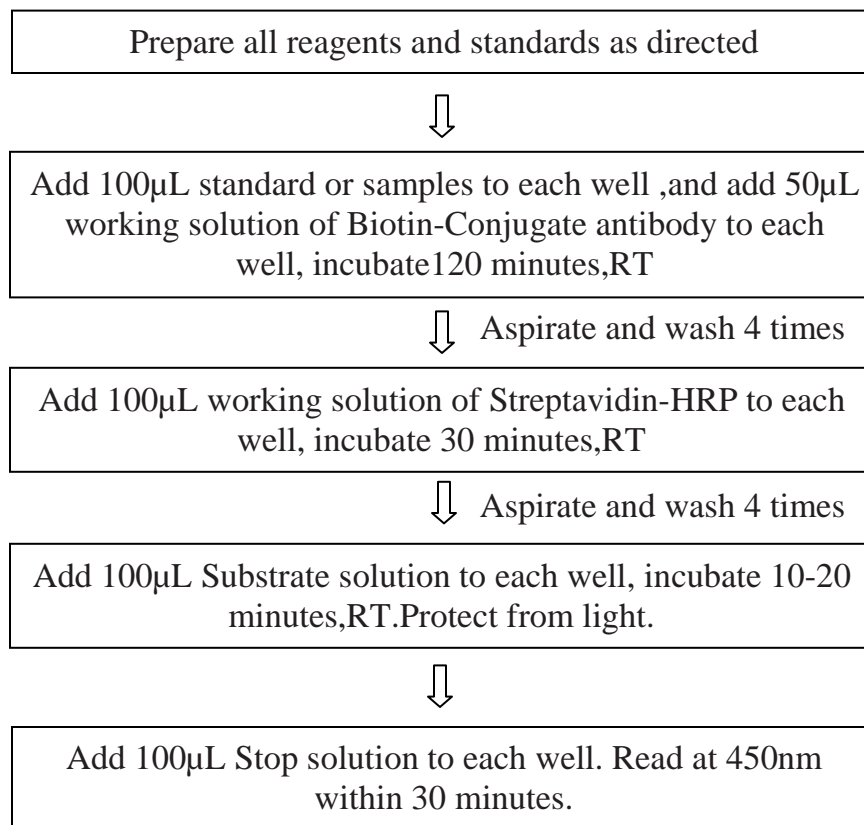


Figure 3: Assay procedure summary

TECHNICAL HINTS

1. When mixing or reconstituting protein solutions, always avoid foaming.
2. 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.
3. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
4. Substrate Solution should remain colorless until added to the plate. Stop Solution should be added to the plate in the same order as the Substrate Solution. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
5. A standard curve should be generated for each set of samples assayed. According to the content of tested factors in the sample, appropriate diluted or concentrated samples, it is best to do pre-experiment.

CALCULATION OF RESULTS

1. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
2. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) 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 the graph.
3. The data may be linearized by plotting the log of the Proinsulin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
4. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Table 3: Typical data using the Proinsulin ELISA (Measuring wavelength:450nm,Reference wavelength:650nm)

Standard (pg/ml)	OD.	OD.	Average	Corrected
0	0.017	0.014	0.016	—
156.25	0.061	0.057	0.059	0.043
312.5	0.107	0.101	0.104	0.088
625	0.196	0.187	0.192	0.176
1250	0.342	0.337	0.340	0.324
2500	0.660	0.652	0.656	0.640
5000	1.145	1.136	1.141	1.125
10000	2.044	2.029	2.037	2.021

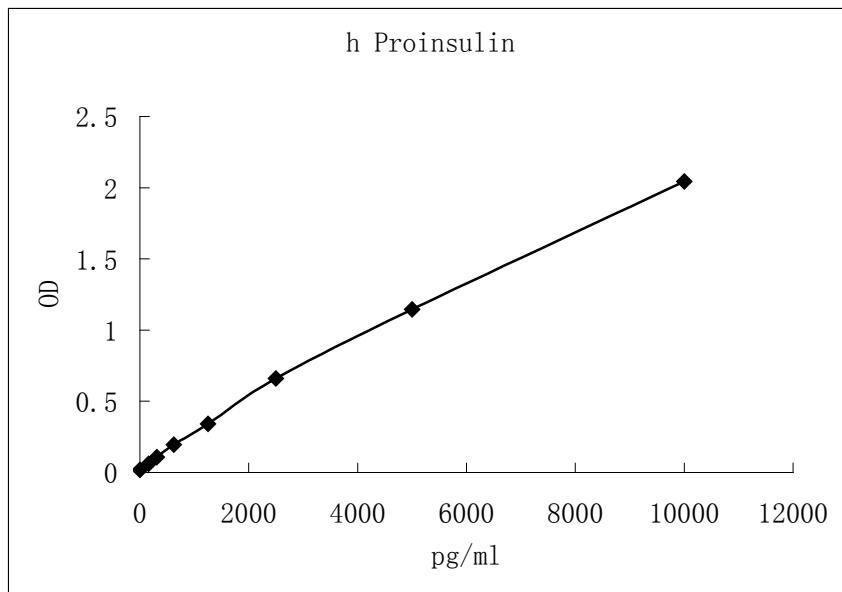


Figure 3: Representative standard curve for Proinsulin ELISA. Proinsulin was diluted in serial two-fold steps in Sample Diluent.

Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

BACKGROUND

Proinsulin is synthesized as a single chain, 110 amino acid (aa)

preproprecursor that contains a 24 aa signal sequence and an 86 aa proinsulin propeptide. Following removal of the signal peptide, the proinsulin peptide undergoes further proteolysis to generate mature insulin, a 51 aa disulfidelinked dimer that consists of a 30 aa B chain (aa 2554) bound to a 21 aa A chain (aa 90110). The 34 aa intervening peptide (aa 5589) that connects the B and A chains is termed the Cpeptide. Human proinsulin shares 84% and 80% aa sequence identity with rat and bovine proinsulin, respectively. Most of the sequence variation between species occurs in the region of the Cpeptide(1). This peptide generates a structural conformation that allows for the correct formation of the intrachain disulphide bonds (1). Insulin is a molecule that facilitates the cellular uptake of glucose. This is accomplished by regulating the appearance of membrane glucose transporters. Low insulin levels or lack of insulin are associated with type 2 and type 1 diabetes mellitus, respectively. These conditions are associated with an increased risk for microvascular complications such as retinopathy, nephropathy, and peripheral neuropathy (3). Proinsulin also circulates, but its physiologic role is less well understood. It does possess about 25% of the activity of mature insulin, but it would seem unlikely to be a natural substitute for insulin (4). In type 2 diabetes, an elevated proinsulin to insulin ratio in the circulation is a well known abnormality (59). Perhaps this abnormality represents either compromised proteolytic processing or a general inability to process increased levels of insulin precursor (5). In any event, proinsulin will stimulate amylin secretion by cells, and amyloid formation in pancreatic islets that promotes decreased cell function (10). Studies also suggest that fasting serum proinsulin may be a better predictor of future type 2 diabetes than fasting insulin levels in obese children (11).

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