



Proinsulin ELISA

For the quantitative determination of proinsulin in serum and plasma

For “In Vitro Diagnostic” use within the United States of America.
This product is for “Research Use Only” outside of the United States of America.

Catalog Number:	80-PINHU-E01.1
Size:	96 wells
Version:	v2.0: June 17, 2010

ALPCO Diagnostics

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INTENDED USE

The ALPCO Proinsulin ELISA is designed for the quantitative determination of proinsulin in serum or plasma.

PRINCIPLE OF THE ASSAY

The ALPCO Proinsulin ELISA is a sandwich type immunoassay. The 96 well microplate is coated with a monoclonal antibody specific for proinsulin. The Proinsulin Standards, Diabetes Controls, and samples are incubated in the microplate wells with Assay Buffer on an orbital microplate shaker at 700-900 rpm. After the first incubation is complete, the wells are washed with Wash Buffer and blotted dry. The horseradish peroxidase enzyme-labeled monoclonal antibody is then added, and the microplate is incubated a second time on an orbital shaker at 700-900 rpm, washed and blotted dry. TMB Substrate is added, and the microplate is incubated a third time on an orbital microplate shaker at 700-900 rpm. Once the third incubation period is complete, Stop Solution is added, and the optical density (OD) is measured by spectrophotometer at 450 nm with a reference wavelength of 620 nm. The intensity of the color generated is directly proportional to the concentration of proinsulin in the sample.

MATERIALS SUPPLIED

Single Plate Kit (80-PINHU-E01.1)

Components	Content	Quantity	Preparation
Proinsulin Microplate	1 microplate	12 x 8 strip wells	Ready to use
Zero Standard	1 vial	5 ml	Ready to use
Proinsulin Standards (2.5, 5, 20, 60, 180 pM)	5 vials	1 ml/vial	Ready to use
Diabetes Controls	2 vials	0.6 ml/vial	Lyophilized
Assay Buffer	1 vial	10 ml	Ready to use
Conjugate Stock	1 vial	1.2 ml	11X
Conjugate Buffer	1 bottle	12 ml	Ready to use
Wash Buffer	1 bottle	40 ml	21X
TMB Substrate	1 bottle	12 ml	Ready to use
Stop Solution	1 bottle	12 ml	Ready to use

MATERIALS REQUIRED BUT NOT SUPPLIED

- Precision pipettes with disposable tips capable of dispensing 25 µl
- Repeating or multi-channel pipette
- Volumetric container
- Volumetric pipettes
- Distilled (deionized) water
- Microplate washer or wash bottle
- Orbital microplate shaker capable of 700-900 rpm
- Microplate reader with 450 and 620-650 nm filter

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency virus), HBV (Hepatitis B virus), and HCV (Human Hepatitis C virus). Test methods for these viruses do not guarantee the absence of virus; therefore, all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. All materials derived from animal sources are BSE negative. However, all materials should be kept from ruminating animals.
3. Avoid direct contact with skin.
4. This product is not for internal use.
5. Avoid eating, drinking, or smoking when using this product.
6. Do not pipette any components by mouth.
7. Components from this kit should not be mixed with components of different lot numbers.
8. Do not use components beyond the expiration date.
9. Variations to the test procedure are not recommended and may influence the test results.

STORAGE CONDITIONS

The kit should be stored at 2-8°C. The kit is stable until the expiration date on the box label. The controls are stable for 7 days at 2-8°C after reconstitution. If desired, the controls can be aliquoted and stored at $\leq -20^{\circ}\text{C}$ until needed. The controls should not be repeatedly frozen and thawed. Working Strength Wash Buffer is stable for 30 days at room temperature (18-25°C).

SAMPLE HANDLING

Serum or plasma (heparin) samples are appropriate for use in this assay. No dilution or treatment of the sample is required. If a sample is $> 180 \text{ pM}$, the sample should be diluted in Zero Standard and the analysis should be repeated.

Samples can be stored at 2-8°C for 24 hours prior to analysis. Storage at $\leq -20^{\circ}\text{C}$ for longer periods is recommended. Avoid repeated freezing/thawing of the sample.

REAGENT PREPARATION

All reagents must reach room temperature prior to preparation and subsequent use in the assay.

Conjugate Stock is diluted with 10 parts Conjugate Buffer. For example, to prepare enough Working Strength Conjugate for one complete plate, dilute 1 ml of Conjugate Stock (11X) with 10 ml of Conjugate Buffer. Prepare an appropriate volume of Working Strength Conjugate immediately before use in the assay.

Diabetes Controls (Levels 1 and 2) are provided in a lyophilized form. Dilute each Control with 0.6 ml of distilled water. Close the vial with the rubber stopper and cap, then gently swirl the vial and allow it to stand for 30 minutes prior to use. The contents should be in solution with no visible particulates. The reconstituted Controls are stable for 7 days stored at 2-8°C. For longer term storage the Controls should be aliquoted and stored at $\leq -20^{\circ}\text{C}$ for up to 6 months (repeated freeze/thaw cycles should be avoided). The concentration ranges of the Controls are

provided on the Certificate of Analysis enclosed with each kit; however, it is recommended that each laboratory establishes its own acceptable ranges.

Wash Buffer is diluted with 20 parts distilled water. For example, to prepare Working Strength Wash Buffer, dilute 20 ml of Wash Buffer (21X) with 400 ml of distilled water. Working Strength Wash Buffer is stable for 30 days at room temperature (18-25°C).

QUALITY CONTROL

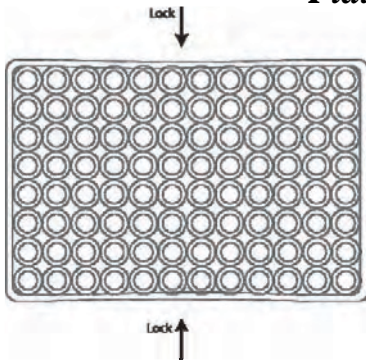
It is recommended that the controls provided with the ALPCO Proinsulin ELISA be included in every assay. The concentration range of the controls is provided on the Certificate of Analysis enclosed with each kit; however, it is recommended that each laboratory establishes its own acceptable range.

ASSAY PROCEDURE

Bring all reagents to room temperature prior to use. Briefly vortex all reagents before use. A standard curve must be included with each assay and/or with each plate, if more than one plate is run at a time. All standards, controls and samples should be run in duplicate.

1. Designate enough microplate–strips for the standards, controls and desired number of samples. The remaining strips should be stored in the tightly sealed foil pouch containing the desiccant and stored at 2-8°C.
2. **Pipette 50 µl** of each Standard, reconstituted Control (see *Reagent Preparation*), or sample into their respective wells.
3. **Pipette 50 µl** of Assay Buffer to each well.
4. **Incubate for 1 hour**, shaking at 700-900 rpm on an orbital microplate shaker at room temperature (18-25°C).
5. **Wash the microplate 6 times** with Working Strength Wash Buffer (see *Reagent Preparation*) with a microplate washer. Alternatively, use a wash bottle to fill the wells and discard the liquid, tapping the plate on absorbent paper between washes. After the final wash, remove any residual Wash Buffer and bubbles from the wells by tapping the plate on absorbent paper towels. (See Plate Locking Diagram below.)
6. **Pipette 100 µl** of Working Strength Conjugate (see *Reagent Preparation*) to each well.
7. **Incubate for 1 hour**, shaking at 700-900 rpm on an orbital microplate shaker at room temperature (18-25°C).
8. **Wash the microplate 6 times** with Working Strength Wash Buffer as in Step 5.
9. **Pipette 100 µl** of Substrate to each well.
10. **Incubate for 15 minutes** at room temperature (18-25°C) on an orbital microplate shaker (700-900 rpm).
11. **Pipette 100 µl** of Stop Solution to each well. Gently mix the wells to stop the reaction. Remove bubbles before reading with microplate reader. The intensity of the yellow color is directly proportional to the concentration of proinsulin in the wells.
12. Place the microplate in a microplate reader capable of reading the absorbance at 450 nm with a reference wavelength of 620-650 nm. The microplate should be analyzed within 30 minutes following the addition of Stop Solution.

Plate Locking Diagram



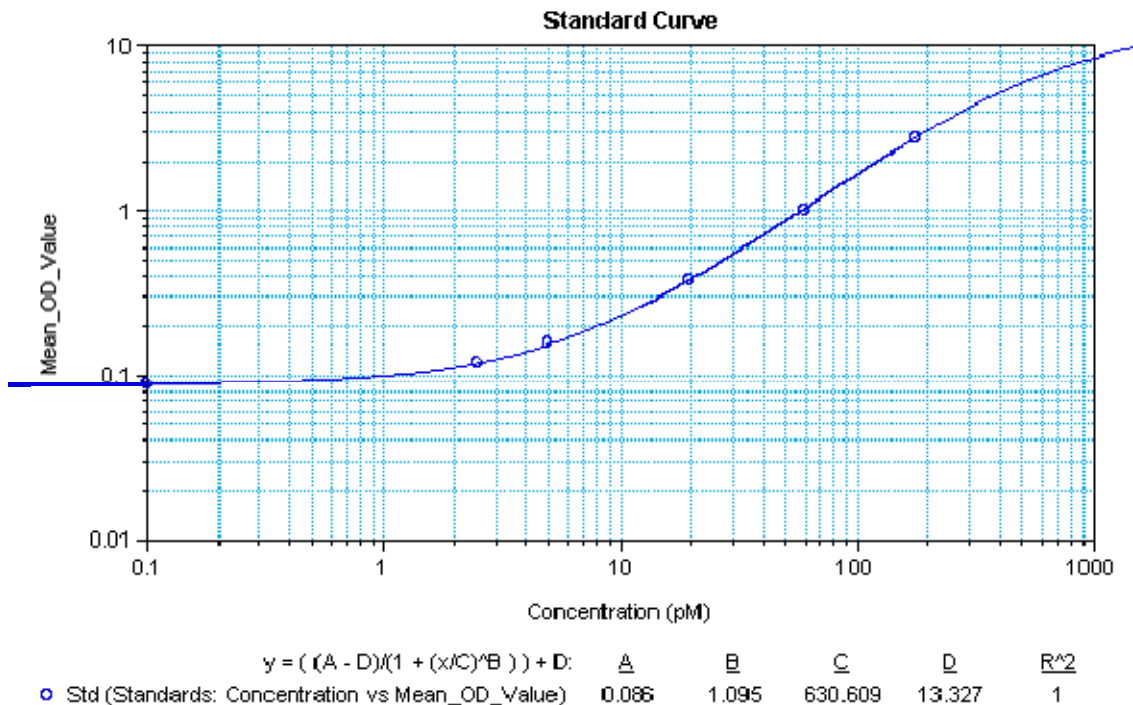
When tapping the microtiter plate onto the absorbent paper, grasp at lock points indicated by the arrows, locking the microwell strips into place. This locking feature allows for tapping with greater force than traditional microtiter plates.

CALCULATION OF RESULTS

Construct a standard curve from the Standards. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the samples.

The Proinsulin ELISA is a ligand binding assay, with responses exhibiting a sigmoidal relationship to the analyte concentration. Currently accepted reference models for such curves use 4 or 5 parameter logistic (pl) fits, as these models optimize the accuracy and precision across a greater range. Although cubic spline and other models are acceptable methods, they generally show less intra-assay accuracy and precision at the low and high ends of the range.

In the example below, a 4 pl curve fit was used to maximize the accuracy and precision of samples with low concentrations (Standard A, 2.5 pM; Zero Standard plotted as 0.1 pM). However, the accuracy and precision of all models are limited at the lowest and highest ends of the detectable range due to the influence of individual laboratory conditions. As a result, caution should always be used when interpreting results where the analyte response becomes non-linear.¹



PERFORMANCE CHARACTERISTICS

Sensitivity:

The analytical sensitivity was determined by calculating the mean \pm 2 standard deviations for 72 replicates of the Zero Standard. The sensitivity of the assay is 1.25 pM.

Precision: Within run (intra-assay) variation

The within run precision is expressed as the percentage coefficient of variation (CV%). This was determined based on the mean and standard deviation of 22 replicates of a sample run in a single assay. The table below shows the results of 4 samples that span the range of the assay.

	Sample 1	Sample 2	Sample 3	Sample 4
mean	5.428 pM	11.977 pM	37.188 pM	59.209 pM
std. dev.	0.209 pM	0.460 pM	2.588 pM	4.000 pM
CV%	3.9	3.8	7.0	6.8
n	22	22	22	22

Precision: Between run (inter-assay) variation

The between run precision is expressed as the percentage coefficient of variation (CV%). This was determined based on the mean and standard deviation across 10 assays of duplicate measurements of a single sample. The table below shows the results of 4 samples that span the range of the assay.

	Sample 1	Sample 2	Sample 3	Sample 4
mean	5.077 pM	12.875 pM	39.064 pM	63.929 pM
std. dev.	0.742 pM	1.200 pM	2.406 pM	4.978 pM
CV%	14.6	9.3	6.2	7.8
n	10	10	10	10

Linearity:

The linearity of the assay was determined by preparing dilutions of 3 samples (shown to have high proinsulin concentrations) with the Zero Standard. The expected values were compared to the obtained values to determine a percent recovery. The range of recovery was 78-118% with an average of 104%.

Spike and Recovery:

The spike and recovery for the assay was determined by adding various known amounts of both Standard E (180 pm) and a WHO reference standard (recombinant human proinsulin at 10 and 20% total volume) to 3 sample pools. These spiked sample pools were evaluated in the assay and the measured concentration was compared to the expected concentration (endogenous + spiked). The range of recovery was 76-100% with an average of 93%.

Specificity:

The table below indicates the analyte and the percent cross-reactivity observed in the assay.

Analyte	% Cross-reactivity
Human C-peptide	< 0.01
Human insulin	0.1
Lispro	0.5

Hook Effect:

No high dose hook effect was observed with proinsulin concentrations up to 18,900 pM.

Accuracy:

The ALPCO Proinsulin ELISA was evaluated for accuracy by comparing its performance to that of another commercially available Proinsulin ELISA. Both methods were used to evaluate 39 samples. The correlation coefficient between the two methods is $r = 0.954$.

REFERENCES

1. Findlay JWA, Dillard RF. Appropriate Calibration Curve Fitting in Ligand Binding Assays. *AAPS Journal*. 2007; 9(2): E260-E267.

Proinsulin ELISA Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

