User Guide

# Human Total Proinsulin

## 96-Well Plate

**EZHPI-15K**

## Intended Use

This Human Total Proinsulin ELISA kit is used for the non-radioactive quantification of human total proinsulin in serum and plasma. This kit has 100% cross reactivity to intact human proinsulin and its major processed intermediate, des(31,32) proinsulin, and 81% cross reactivity to its processed intermediate des(64,65) proinsulin in serum and plasma. Human Insulin (up to 200 µU/mL) and Human C-Peptide (up to 10 ng/mL) do not interfere with the assay result. One kit is sufficient to measure 38 unknown samples in duplicate. This kit has no cross reactivity to human insulin.

**This kit is for Research Use Only. Not for Use in Diagnostic Procedures.**

## Principles of Assay

This assay is a Sandwich ELISA based, sequentially, on:

- Capture of human proinsulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of polyclonal guinea pig anti-human insulin antibodies
- Wash away of unbound materials from samples
- Binding of a second biotinylated monoclonal mouse anti-human antibody to the c-peptide section of the captured molecules
- Wash away of unbound materials from samples
- Conjugation of horseradish peroxidase to the immobilized biotinylated antibodies
- Wash away of free enzyme
- Quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3’,5,5’-tetra-methylbenzidine.

The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human total proinsulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human proinsulin.

## Reagents Supplied

Each kit is sufficient to run one 96-well plate and contains the following reagents:

**Note:** Store all reagents at 2-8 °C.

| Reagents Supplied | Volume | Quantity | Cat. No. |
|---|---|---|---|
| Human Proinsulin ELISA Plate with 2 plate sealers<br>**Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2–8 °C | - | 1 plate<br>2 sealers | EP15 |
| Human Proinsulin Standards<br>Human Proinsulin in Buffer:<br>2, 5, 10, 20, 50, 100 and 200 pM.<br>**Note:** The standard(s) in this kit have been calibrated to an International Reference standard, NIBSC code # 84/611. | 0.5 mL | 1 bottle each | E8015-K |
| ELISA Quality Controls 1 and 2 | 0.5 mL | 1 bottle each | E6000-K |
| Matrix Solution<br>Charcoal stripped Proinsulin Depleted Human Serum | 1 mL | 1 vial | EMTX |
| Assay Buffer<br>0.025 M Phosphosaline, pH 6.8, containing 0.025 M EDTA, 0.08% Sodium azide, and 1% BSA | 9 mL | 1 vial | EABU |
| 10X HRP Wash Buffer Concentrate.<br>10X concentrate of 50 mM Tris Buffered Saline containing Tween®-20 | 50 mL | 2 bottles | EWB-HRP |
| Human Total Proinsulin Detection Antibody | 12 mL | 1 vial | E1015 |
| Enzyme Solution | 12 mL | 1 vial | EHRP |
| Substrate Solution<br>3,3’,5,5’-tetremethylbenzidine in buffer<br>**Note:** Minimize light exposure. | 12 mL | 1 vial | ESS-TMB |
| Stop Solution<br>0.3 M HCl **Caution:** Corrosive Solution | 12 mL | 1 vial | ET-TMB |

## Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Once opened, liquid standards and controls can be stored up to 30 days at 2-8°C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

## Reagent Precautions

### Sodium Azide

Sodium azide or Proclin™ has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide and Proclin™ may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

### Hydrochloric Acid

Hydrochloric acid is corrosive and can cause eye and skin burns. Harmful if swallowed. Causes respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

**Note:** See next page for Hazardous Components' full labels.

## Symbol Definitions

| Ingredient | Cat. No. | Full Label | |
|---|---|---|---|
| Enzyme Solution | ET-TMB |  | **Danger:** May be corrosive to metals. |
| 10X HRP Wash Buffer Concentrate | EWB-HRP |  | **Warning:** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water. |

## Materials Required (Not Provided)

- Multi-channel Pipettes and pipette tips: 5 µL-50 µL and 50 µL-300 µL
- Pipettes and pipette tips: 10 µL-20 µL or 20 µL-100 µL
- Buffer and Reagent Reservoirs
- Vortex Mixer
- De-ionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm and 590 nm
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth

## Sample Collection and Storage

1. To prepare serum samples, whole blood is directly drawn into a Vacutainer® serum tube that contains no anticoagulant. Let blood clot at room temperature for 30 min.
2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x *g* for 15 minutes at 4 ±2 °C.
3. Transfer and store serum samples in separate tubes. Date and identify each sample.
4. Use freshly prepared serum or store samples in aliquots at ≤ –20 °C for later use. Avoid freeze/thaw cycles.
5. To prepare plasma samples, whole blood should be collected into Vacutainer® EDTA-plasma tubes and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.
6. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.
7. Avoid using samples with gross hemolysis or lipemia.

## Assay Procedure

Pre-warm all reagents to room temperature before setting up the assay.

1. Dilute the 10X Wash Buffer concentrate 10-fold by mixing the entire content of each bottle of Wash Buffer with 450 mL deionized water (dilute both bottles with 900 mL deionized water).
2. Remove the required number of strips from the Microtiter Assay Plate. Assemble the strips in an empty plate holder fill each well with 300 µL of diluted HRP Wash Buffer. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 3 times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.
3. Add 60 µL Assay Buffer to the Standard and Quality Control wells.
4. Add 80 µL Assay Buffer to each of the NSB and sample wells.
5. Add 20 µL Matrix Solution to the NSB, Standard, and Quality Control wells (refer to Microtiter Plate Arrangement for suggested well orientations).
6. Add in duplicate 20 µL Human Proinsulin Standards in the order of ascending concentration to the appropriate wells.
7. Add in duplicate 20 µL QC1 and 20 µL QC2 to the appropriate wells.
8. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells. For best result all additions should be completed within one hour. Cover the plate with plate sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm).
9. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.
10. Wash wells 3 times with diluted HRP Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.
11. Transfer Detection Antibody solution to a reagent reservoir and add 100 µL of this solution to each well with a multi-channel pipette. Cover the plate with plate sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm).
12. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells.
13. Wash wells 3 times with diluted HRP Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.
14. Add 100 µL Enzyme Solution to each well. Cover the plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

15. Remove sealer, decant solutions from the plate, and tap plate to remove the residual fluid.

16. Wash wells 6 times with diluted HRP Wash Buffer, 300 µL per well per wash. Decant and tap firmly after each wash to remove residual buffer.

17. Add 100 µL of substrate solution to each well, cover plate with sealer and shake on the plate shaker for approximately 15-20 minutes.

    **Note:** Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

18. Remove sealer and add 100 µL Stop Solution (**Caution:** Corrosive Solution) and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. If wavelength correction is available, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 590, 600, or 620 nm. If the absorbance readings exceed the limitations of the plate reader, a second reading at 405 nm is needed (reference filter 590, 600 or 620 if available). In this case, proceed to construct a second standard curve as above with the absorbance readings of all Standards at 405 nm. The concentrations of the off-scale samples at 450 nm are than read from the new standard curve. The readings at 405 nm should not replace the on-scale readings at 450 nm.

# Microtiter Plate Arrangement

**Standard Human Proinsulin ELISA**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blank | 20 pM | QC 1 | Etc. | | | | | | | | |
| B | Blank | 20 pM | QC 1 | Etc. | | | | | | | | |
| C | 2 pM | 50 pM | QC 2 | | | | | | | | | |
| D | 2 pM | 50 pM | QC 2 | | | | | | | | | |
| E | 5 pM | 100 pM | Sample 1 | | | | | | | | | |
| F | 5 pM | 100 pM | Sample 1 | | | | | | | | | |
| G | 10 pM | 200 pM | Sample 2 | | | | | | | | | |
| H | 10 pM | 200 pM | Sample 2 | | | | | | | | | |

## Calculations

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

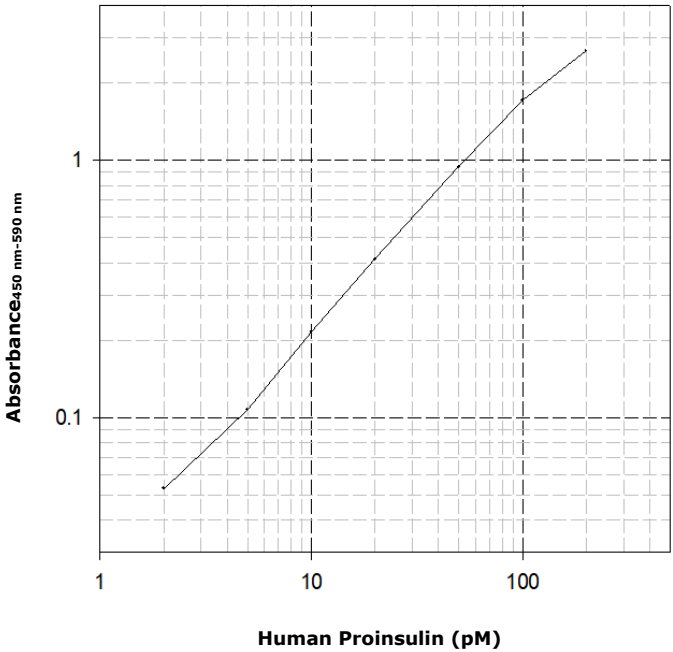
**Note:** When sample volumes assayed differ from 20 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (for example, if 10 µL of sample is used, then observed data must be multiplied by 2). When sample volume assayed is less than 20 µL, compensate the volume deficit with matrix solution.

## Interpretation

- The run will be considered accepted when all Quality Control values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review results with the supervisor.
- If the difference between duplicate results of a sample is > 15% CV, repeat the sample.
- The limit of sensitivity of this assay is 0.5 pM human proinsulin (20 µL sample size).
- The dynamic range of this assay is 2 pM to 200 pM human proinsulin (20 µL sample size). Any result greater than 200 pM in a 20 µL sample should be diluted using matrix solution as diluent, and the assay repeated until the results fall within range.

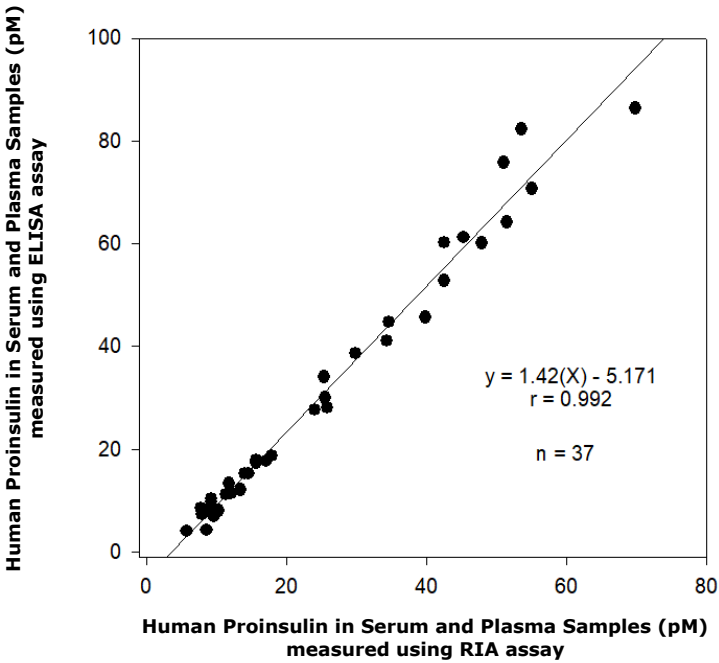
# Standard Curve and Correlation Graph

**Human Proinsulin ELISA**




For Reference Only – Typical Standard Curve

## Human Proinsulin ELISA vs RIA Correlation




Serum samples obtained from 37 human subjects were assayed for proinsulin content using both Human Proinsulin RIA Kit (Catalogue #HPI-15K) and Human Total Proinsulin ELISA Kit (EZHPI-15K). Correlation of the two kits are derived by linear regression analysis of paired results from each sample.

# Assay Characteristics

## Sensitivity

The lowest level of Proinsulin that can be detected by this assay is 0.5 pM when using a 20 µL sample size.

## Specificity

The specificity (also known as selectivity) of the analytical test is its ability to selectively measure the analytes in the presence of other like components in the sample matrix.

| | |
|---|---|
| Human Insulin | n.d.* |
| Intact Human Proinsulin | 100% |
| Des(64,65) Human Proinsulin | 81% |
| Des(31,32) Human Proinsulin | 100% |
| Porcine Proinsulin | ~1% |
| Bovine Proinsulin | n.d.* |
| Glucagon | n.d.* |
| Human C-peptide | n.d.* |
| Rat C-peptide | n.d.* |
| Human Leptin | n.d.* |
| Rat Leptin | n.d.* |

n.d.: not detectable at concentrations up to * 20 nM

## Precision

**Intra- and Inter-Assay Variation**

| Sample No. | Mean Proinsulin Levels (pM) | Intra % CV | Inter % CV |
|---|---|---|---|
| 1 | 15.15 | 6.3 | 3.0 |
| 2 | 80.63 | 0.8 | 2.9 |
| 3 | 6.52 | 8.5 | 8.3 |

The assay variations of Human Total Proinsulin ELISA kits were studied on three human serum samples with varying concentrations of endogenous proinsulin. The mean within variation was calculated from results of six duplicate determinations in each assay of the indicated samples. The mean between variation of each sample was calculated from results of four separate assays with duplicate samples in each assay.

## Spike & Recovery of Human Proinsulin in Serum

| Sample No. | Proinsulin Added (pM) | Expected (pM) | Observed (pM) | % of Recovery |
|---|---|---|---|---|
| 1 | 0 | 16.1 | 16.1 | 100 |
| | 10 | 26.1 | 26.1 | 100 |
| | 50 | 66.1 | 63.7 | 96.4 |
| | 100 | 116.1 | 108.8 | 93.7 |
| 2 | 0 | 82.4 | 82.4 | 100 |
| | 10 | 92.4 | 91.4 | 98.9 |
| | 50 | 132.4 | 119.3 | 90.3 |
| | 100 | 182.4 | 160.9 | 88.2 |
| 3 | 0 | 7.5 | 7.5 | 100 |
| | 10 | 17.5 | 18 | 103 |
| | 50 | 57.5 | 56.3 | 97.9 |
| | 100 | 107.5 | 100.8 | 93.8 |

Varying amounts of human proinsulin were added to three human serum samples and the proinsulin content was determined in four separate assays.

The % of recovery = observed proinsulin concentrations/expected proinsulin concentrations x 100%.

## Linearity of Sample Dilution

| Sample No. | Volume Sampled (µL) | Expected (pM) | Observed (pM) | % of Expected |
|---|---|---|---|---|
| 1 | 20 | 52.8 | 52.8 | 100 |
| | 10 | | 53.4 | 101 |
| | 4 | | 52.5 | 100 |
| | 2 | | 64 | 120 |
| 2 | 20 | 85.9 | 85.9 | 100 |
| | 10 | | 86 | 100 |
| | 4 | | 81.5 | 95 |
| | 2 | | 82 | 95 |
| 3 | 20 | 17.9 | 17.9 | 100 |
| | 10 | | 18.4 | 103 |
| | 4 | | 16 | 89 |
| | 2 | | 15 | 84 |

Three human serum samples with the indicated sample volumes were assayed in four separate experiments. Required amounts of assay buffer or matrix solution were added to compensate for lost volumes below 20 µL. The resulting dilution factors of 1.0, 2.0, 5.0, and 10.0 representing 20 µL, 10 µL, 4 µL, and 2 µL sample volumes assayed, respectively, were applied in the calculation of observed proinsulin concentrations.

% expected = observed/expected x 100%.

## Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

## Troubleshooting

- To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
- Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
- Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
- Avoid cross contamination of any reagents or samples to be used in the assay.
- Make sure all reagents and samples are added to the bottom of each well.
- Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
- Remove any air bubbles formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
- Do not let the absorbency reading of the highest standard reach 3.0 units or higher after acidification.
- High absorbance in background or blank wells could be due to
    - cross well contamination by standard solution or sample
    - inadequate washing of wells with Wash Buffer
    - overexposure to light after substrate has been added.

# Product Ordering

Products are available for online ordering at SigmaAldrich.com.

## Replacement Reagents

| Reagents | Cat. No. |
|---|---|
| Human Proinsulin ELISA Plate | EP15 |
| 10X HRP Wash Buffer Concentrate | EWB-HRP |
| Human Proinsulin Standards | E8015-K |
| ELISA Quality Controls 1 & 2 | E6000-K |
| Matrix Solution | EMTX |
| Assay Buffer | EABU |
| Human Total Proinsulin Detection Antibody | E1015 |
| Enzyme Solution | EHRP |
| Substrate | ESS-TMB |
| Stop Solution | ET-TMB |
| 10-pack of Human Total Proinsulin ELISA Kits | EZHPI-15BK |

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