User Guide

Human Pancreatic Polypeptide (PP) ELISA Kit

96-Well Plate

EZHPP40K
EZHPP-40BK

Intended Use

This Human Pancreatic Polypeptide (PP) ELISA kit is used for the non-radioactive quantification of Human PP in human serum, plasma, tissue extract and cell culture samples. One kit is sufficient to measure 39 unknown samples in duplicate.

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 PP molecules from samples to the wells of a microtiter plate coated with a pre-tittered amount of anti-human PP polyclonal antibody
- Washing of unbound materials from samples
- Binding of a second biotinylated anti-PP polyclonal antibody to the captured molecules
- Washing of unbound materials from samples,
- Binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated antibodies
- Washing of excess free enzyme conjugates
- Quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3’,5,5’-tetramethylbenzidine

The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm–590 nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured human PP 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 PP.

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.
Microtiter Plate with 2 plate sealers	-	1 plate 2 sealers	EP40
Human PP Standard	0.5 mL/vial Lyophilized	1 vial	E8040K
Human PP Quality Controls 1 and 2	0.5 mL/vial Lyophilized	1 vial each	E6040K
Serum Matrix	1 mL/vial Lyophilized	1 vial	EMTXPP
Assay Buffer	40 mL	-	EAB-P
10X Wash Buffer	50 mL	2 bottles	EWB-HRP
Human PP Detection Antibody	12 mL	1 bottle	E1040
Enzyme Solution	12 mL	1 bottle	EHRP-3
Substrate Solution	12 mL	1 bottle	ESS-TMB
Stop 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. Reconstituted standards and controls can be frozen for future use but repeated freeze/thaw cycles should be avoided. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

Symbol Definitions

Ingredient	Cat No.	Full Label	
Human Pancreatic Polypeptide Detection Antibody	E1040		**Warning**. Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Human Pancreatic Polypeptide Quality Control 1 & 2	E6040K		**Danger**. Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention.
Human Pancreatic Polypeptide Standard	E8040K		**Danger**. Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Assay Buffer	EAB-P		**Warning**. Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Stop Solution	ET-TMB		**Warning**. 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.

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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. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes. Do not swallow or ingest.

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
- Reagent Reservoirs
- Polypropylene Microfuge Tubes
- Vortex Mixer
- De-ionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth

Sample Collection and Storage

Preparation of Serum Samples

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. 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 aliquot and store samples at ≤ −20 °C for later use. For long-term storage, keep at −70 °C.
5. Avoid freeze/thaw cycles.
6. No dilution or preparation is needed for normal serum or plasma samples. In the event that any sample is above 3000 pg/mL range, dilutions should be performed using the Serum Matrix provided.
7. Tissue extracts or cell culture media samples may require dilution. Dilutions should be performed using the assay buffer provided.

Preparation of Plasma Samples

1. To prepare plasma samples, whole blood should be collected into centrifuge tubes containing enough K_3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.
2. 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.
3. No dilution or preparation is needed for normal serum or plasma samples. In the event that any sample is above 3000 pg/mL range, dilutions should be performed using the Serum Matrix provided. Avoid using samples with gross hemolysis or lipemia.
4. Tissue extracts or cell culture media samples may require dilution. Dilutions should be performed using the assay buffer provided.

Preparation of Tissue Culture Supernatant

1. Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ −20 °C.
2. Avoid multiple (> 2) freeze/thaw cycles.
3. Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. Tissue/cell extracts should be done in neutral buffers containing reagents and conditions that do not interfere with assay performance. Excess concentrations of detergent, salt, denaturants, high or low pH, etc. will negatively affect the assay. Organic solvents should be avoided. The tissue/cell extract samples should be free of particles such as cells or tissue debris.

Note:

- A maximum of 25 µL per well of neat serum or plasma can be used. Tissue culture or other media may also be used.
- Tissue extracts or cell culture media samples may require dilution. Dilutions should be performed using the assay buffer provided.
- All samples must be stored in polypropylene tubes. DO NOT STORE SAMPLES IN GLASS.
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anti-coagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

Reagent Preparation

Human PP Standard Preparation

Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human PP Standard with 0.5 mL distilled or de-ionized water. Invert and mix gently, let sit for 5 minutes then mix well.

1. Label 5 polypropylene microfuge tubes as 1, 2, 3, 4 and 5.
2. Add 200 µL of Assay Buffer to each of the five tubes.
3. Prepare 3 times serial dilutions by adding 100 µL of the reconstituted standard to the tube 1, mix well.
4. Transfer 100 µL of the tube 1 standard to the tube 2, mix well.
5. Transfer 100 µL of the tube 2 standard to the tube 3, mix well.
6. Transfer 100 µL of the tube 3 standard to the tube 4, mix well.
7. Transfer 100 µL of the tube 4 standard to the tube 5, mix well
8. The 0 µL standard (Background) will be Assay Buffer.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with standard before dispensing. Unused portions of reconstituted standard should be stored in small aliquots at ≤ −20 °C. Avoid multiple freeze/thaw cycles.

Tube #	Volume of Deionized Water to Add	Volume of Standard to Add	Standard Stock Concentration
Reconstituted standard	0.5 mL	0	X (refer to analysis sheet for exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration (pg/mL)
Tube 1	200 µL	100 µL of reconstituted standard	X/3
Tube 2	200 µL	100 µL of Tube 1	X/9
Tube 3	200 µL	100 µL of Tube 2	X/27
Tube 4	200 µL	100 µL of Tube 3	X/81
Tube 5	200 µL	100 µL of Tube 4	X/243

Human PP Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute Human PP Quality Control 1 and Quality Control 2 with 0.5 mL distilled or de-ionized water and Invert and mix gently, let sit for 5 minutes then mix well to ensure complete hydration.

Preparation of Matrix Solution

Use care in opening the lyophilized Matrix Solution. Using a pipette, reconstitute the Matrix Solution with 1.0 mL distilled or deionized water. Invert and mix gently, let sit for 5 minutes then mix well.

Assay Procedure

Warm all reagents to room temperature before setting up the assay.

1. Dilute the 10X concentrated Wash Buffer 10-fold by mixing the entire content of each bottle of wash buffer concentrate with 450 mL deionized or distilled water (dilute both buffer bottles with 900 ml deionized or distilled water).
2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8 °C. Assemble the strips in an empty plate holder. Add 300 µL diluted Wash Buffer to each well of the plate. Incubate at room temperature for 5 minutes. Decant Wash Buffer and remove the residual volume by inverting the plate and tapping it smartly onto absorbent towels several 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 in duplicate, 50 µL Assay Buffer to the blank wells and sample wells.
4. Add 50 µL Matrix Solution to Blank wells, Standard wells, and QC1 and QC2 wells.
5. Add in duplicate, 50 µL Human PP Standards in the order of ascending concentration to the appropriate wells. Add in duplicate, 50 µL QC1 and 50 µL QC2 to the appropriate wells. Add sequentially, 50 µL of the unknown samples in duplicate to the remaining wells. For best result all additions should be completed within 30 minutes.
6. Cover the plate with plate sealer and incubate at room temperature for 1.5 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
7. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.
8. Add 100 µL Detection Antibody to each well. Re-cover plate with sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400-500 rpm.
9. Remove plate sealer and decant reagents from the plate. Tap as before to remove residual volume in wells. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.
10. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.
11. Remove sealer, decant reagents from the plate and tap plate to remove the residual volume. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

12. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 5 to 20 minutes. (A longer development time may be needed if using a plate washer). Blue color should be formed in wells of the PP standards with intensity proportional to increasing concentrations of PP.

    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.

13. Remove sealer and add 100 µL Stop Solution (**Caution:** Corrosive Solution) and gently 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 and 590 nm in a plate reader within 5 minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units. The absorbance of the highest PP standard should be approximately 2.0-3.2, or not to exceed the capability of the plate reader used.

Assay Procedure for Human PP ELISA Kit

Well #	Step 1	Step 2 Matrix Solution	Step 3 Assay Buffer	Step 4 Standards/ QCs/Samples	Step 5-6	Step 7 Detection Antibody	Step 8	Step 9 Enzyme Solution	Step 10	Step 11-12 Substrate	Step 11-12 Stop
A1, B1	Wash plate with 300 µL 1X Wash Buffer. Incubate for 5 minutes. Remove residual buffer by tapping smartly on absorbent towels.	50 µL	50 µL	-	Seal, agitate, incubate 1.5 hours at room temperature on a plate shaker. Wash 3X with 300 µL Wash Buffer.	100 µL	Seal, agitate, incubate 1 hour at room temperature on a plate shaker. Wash 3X with 300 µL Wash Buffer.	100 µL	Seal, agitate, incubate 30 minutes at room temperature on a plate shaker. Wash 3X with 300 µL Wash Buffer.	100 µL	100 µL
C1, D1		50 µL	-	50 µL of Tube 5							
E1, F1		50 µL	-	50 µL of Tube 4							
G1, H1		50 µL	-	50 µL of Tube 3							
A2, B2		50 µL	-	50 µL of Tube 2							
C2, D2		50 µL	-	50 µL of Tube 1							
E2, F2		50 µL	-	50 µL of reconstituted std.							
G2, H2		50 µL	-	50 µL of QC 1							
A3, B3		50 µL	-	50 µL of QC 2							
C3, D3		-	50 µL	50 µL of sample							
E3, F3		-	50 µL	50 µL of Sample							
G3, H3, etc.		-	50 µL	50 µL of sample							

Seal, Agitate, Incubate for 5-20 minutes at room temperature.

Read Absorbance at 450 nm and 590 nm.

Microtiter Plate Arrangement

Human Pancreatic Polypeptide (PP) ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 2	QC 2									
B	Blank	Tube 2	QC 2									
C	Tube 5	Tube 1	Sample									
D	Tube 5	Tube 1	Sample									
E	Tube 4	Reconstituted Standard	Sample									
F	Tube 4	Reconstituted Standard	Sample									
G	Tube 3	QC 1	Etc.									
H	Tube 3	QC 1	Etc.									

Calculations

The dose-response curve of this assay fits best to a 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 50 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (For example, if 25 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 50 µL, compensate the volume deficit with matrix solution.

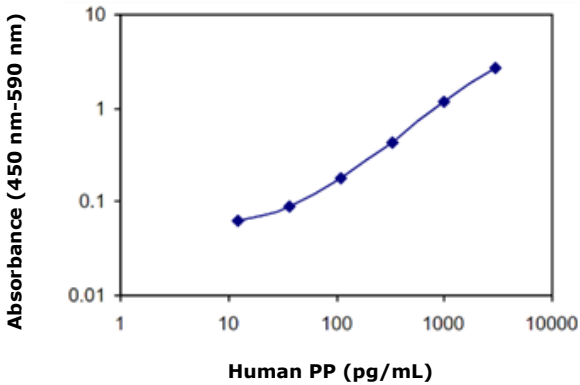
Interpretation

Acceptance Criteria

1. The assay 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 a supervisor.
2. If the difference between duplicate results of a sample is > 10% CV, repeat the sample.
3. The limit of sensitivity of this assay is 12.3 pg/mL human PP (50 µL sample size).
4. The appropriate range of this assay is 12.3 pg/mL to 3000 pg/mL human PP (50 µL sample size). Any result greater than 3000 pg/mL in a 50 µL sample should be diluted using matrix solution, and the assay repeated until the results fall within range. Tissue/cell extracts or cell culture media samples greater than 3000 pg/mL in a 50 µL sample should be diluted in Assay Buffer.

Assay Characteristics

Graph of Typical Reference Curve



Typical Standard Curve, not to be used to calculate data.

Sensitivity

The lowest level of Human PP that can be detected by this assay is 12.3 pg/mL when using a 50 µL sample size.

Specificity

The antibody pair used in this assay is specific to Human PP and has no significant cross-reactivity with NPY, PYY, and other gut hormones. This assay does not recognize Rat PP.

Precision

The assay variations of our Human PP ELISA kit were studied on six human serum samples at varying concentrations of endogenous PP on the Human PP ELISA standard curve. The mean intra-assay variation was calculated from results of six duplicate determinations in each assay of the indicated samples. The mean inter-assay variations of each sample were calculated from results of four separate assays with duplicate samples in each assay.

Intra-Assay Variation

	Mean PP Levels (pg/mL)	Intra-Assay %CV
1	114	3.3%
2	82	4.2%
3	86	4.7%
4	893	5.0%

Inter-Assay Variation

	Mean PP Levels (pg/mL)	Inter-Assay %CV
1	103	9.8%
2	74	5.5%
3	812	4.4%
4	128	4.9%

Spike Recovery of Human PP in Assay Samples

Varying amounts of Human PP were added to three human serum samples and the PP concentration was determined in three separate assays.
The % of recovery = observed PP concentrations/expected PP concentrations x 100%.

Sample	PP Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery
1	0	14	14	-
	111.1	125.1	107	86%
	333.3	347.3	284	82%
	1000	1014	806	79%
2	0	58	58	-
	111.1	169.1	158	93%
	333.3	391.3	370	95%
	1000	1058	1002	95%
3	0	14	2	-
	111.1	125.1	108	95%
	333.3	347.3	289	86%
	1000	1014	828	83%

Linearity of Sample Dilution

Three human serum samples with the indicated sample volumes were assayed. Required amounts of matrix were added to compensate for lost volumes below 50 µL. The resulting dilution factors of neat, 2, 5 and 10 representing 50 µL, 25 µL, 10 µL and 5 µL sample volumes assayed, respectively, were applied in the calculation of observed PP concentrations.

% expected = (observed/expected) x 100%

Sample	Volume (µL)	Expected (pg/mL)	Observed (pg/mL)	Expected
1	50	760	760	-
	25	380	351	92%
	10	152	148	97%
	5	76	84	111%
2	50	958	958	-
	25	479	428	89%
	10	191.6	181	94%
	5	95.8	114	119%
3	50	227	227	-
	25	113.5	98	86%
	10	45.4	41	90%
	5	22.7	23	101%

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.
- High signal in background or blank wells could be due to:
    - cross well contamination by standard solution or sample, or
    - inadequate washing of wells with Wash Buffer, or
    - 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 PP ELISA Plate	EP40
10X HRP Wash Buffer Concentrate	EWB-HRP
Human PP ELISA Standard	E8040K
Human PP Quality Controls 1 and 2	E6040K
Matrix Solution	EMTXPP
Assay Buffer	EAB-P
Human PP Detection Antibody	E1040
Enzyme Solution	EHRP-3
Substrate Solution	ESS-TMB
Stop Solution	ET-TMB
10-pack of Human Pancreatic Polypeptide (PP) ELISA kits	EZHPP-40BK

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