User Guide

Human PIIANP

96-Well Plate

EZPIIANP-53K

Intended Use

Type II collagen is the major collagen found in cartilage and is expressed in two forms: IIA and IIB. Type IIA procollagen contains an N-terminal 69 amino acid, cysteine-rich globular domain that is encoded by exon 2. Type IIB procollagen is synthesized by mature chondrocytes in cartilage while Type IIA procollagen is synthesized by chondroprogenitor cells. The Type IIA N-propeptide (PIIANP) has been postulated to play a role in chondrogensis. Type IIA procollagen has been found to be synthesized by osteoarthritic chondrocytes in diseased cartilage and may serve as a specific arthritis biomarker that reflects an attempt by the chondrocytes to repair diseased cartilage.

This kit is used for the quantification of Type IIA collagen N-Propeptide (PIIANP) in human serum. Plasma samples are incompatible with this assay and application to samples of other biological fluids may need validation by the user. One kit is sufficient to measure 38 unknown samples in duplicate.

This kit is for research use only. Not for use in diagnostic procedures.

Principles of Assay

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

- Binding of PIIANP in the sample to pre-titered antiserum while in the presence of competing biotinylated PIIANP peptide and the immobilization of the resulting complexes in the wells of a microtiter plate
- After washing, binding of horseradish peroxidase to the immobilized biotinylated PIIANP
- Wash away of 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 absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is inversely proportional to the amount of captured PIIANP 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 PIIANP.

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 **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided.	-	1 plate 2 sealers	EP53
Anti-PIIANP Antibody	6 mL	1 vial	E1053
PIIANP Standard	Lyophilized	1 vial	E8053-K
Quality Controls 1 and 2 Each vial contains PIIANP at different levels	Lyophilized	1 vial each	E6053-K
Assay Buffer 0.01 M phosphate buffer, pH 7.4, containing 0.1% BSA, sodium azide, 0.025% Tween®-20	25 mL	1 vial	EABPT
10X Wash Buffer 10X concentrate of 50 mM Tris Buffered Saline containing Tween®-20	50 mL	2 bottles	EWB-HRP
Biotin Labelled PIIANP	Lyophilized	1 vial	EBT53
Enzyme Solution Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.	12 mL	1 bottle	EHRP-53
Substrate Solution 3,3’,5,5’-tetramethylbenzidine in buffer (Light Sensitive: avoid unnecessary exposure to light)	12 mL	1 bottle	ESS-TMB2
Stop Solution 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. 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.

Reagent Precautions

Sodium Azide

Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide 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 eye. Do not swallow or ingest.

Note: See next page for Hazardous Component full labels.

Symbol Definitions

Ingredient	Cat. No.	Full Label	
PIIANP Quality Controls 1 and 2	E6053-K		**Danger:** Toxic if swallowed. Very toxic to aquatic life with long lasting effects. Avoid release to the environment. IF exposed or concerned: immediately call a POISON CENTER or doctor/ physician.
PIIANP Standard	E8053-K		**Danger:** Toxic if swallowed. Very toxic to aquatic life with long lasting effects. Avoid release to the environment. IF exposed or concerned: immediately call a POISON CENTER or doctor/physician.
Biotin Labeled PIIANP	EBT53		**Danger:** Toxic if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF exposed or concerned: immediately call a POISON CENTER or doctor/ physician.
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.

Materials Required (Not Provided)

- Multi-channel Pipettes and pipette tips: 0 µL-50 µL and 50 µL-300 µL
- Pipettes and pipette tips: 5 µL-20 µL, 20 µL-100 µL, 1000 µL–5000 µL
- Buffer and Reagent Reservoirs
- Vortex Mixer
- Deionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth

Sample Collection and Storage

1. To prepare human 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. Avoid multiple (> 3) freeze/thaw multiple cycles.
5. Avoid using samples with gross hemolysis or lipemia.

Reagent Preparation

Preparation of Biotin-labeled PIIANP

Rehydrate the provided vial of Biotin-labeled PIIANP with 5.0 mL of Assay Buffer. Assuring that the stopper is securely on the vial, gently invert the vial and mix the contents thoroughly. Let the contents of the bottle sit for at least 5 minutes prior to setting up the assay.

Note: If only a partial plate is used, you may freeze the remaining biotin-labeled PIIANP at −20 °C for future use. To do this, transfer the remaining solution to polypropylene tube. Allow to thaw completely and vortex well prior to performing the next assay. Avoid multiple freeze/ thaw cycles.

PIIANP Standard Preparation

6. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the PIIANP standard with 0.2 mL deionized water to give a concentration prescribed in analysis sheet. Invert and mix gently, let sit for 5 minutes then mix well.
7. Label six tubes 1, 2, 3, 4, 5, and 6. Add 50 µL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 µL of the reconstituted standard to tube 1, mix well and transfer 50 µL of tube 1 to tube 2, mix well and transfer 50 µL of tube 2 to tube 3, mix well and transfer 50 µL of tube 3 to tube 4, mix well and transfer 50 µL of tube 4 to tube 5, mix well and transfer 50 µL of tube 5 to tube 6, and mix well.

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

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration ng/mL
200 µL	0	X (Refer to analysis sheet for exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration ng/mL
1	50 µL	50 µL of reconstituted standard	X/2
2	50 µL	50 µL of tube 1	X/4
3	50 µL	50 µL of tube 2	X/8
4	50 µL	50 µL of tube 3	X/16
5	50 µL	50 µL of tube 4	X/32
6	50 µL	50 µL of tube 5	X/64

PIIANP Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the PIIANP Quality Control 1 and Quality Control 2 with 0.2 mL distilled or deionized water. Invert and mix gently, let sit for 5 minutes then mix well.

Note: For exact ranges of Quality Control 1 and 2, refer to Analysis Sheet. Unused portions of the reconstituted Quality Controls should be stored at ≤ −20 °C. Avoid multiple freeze/thaw cycles.

Assay Procedure

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

1. Dilute the 10X concentrated HRP Wash Buffer 10-fold by mixing the entire contents of both buffer bottles with 900 mL de-ionized or distilled water.

8. Remove the required number of strips from the Microtiter Assay Plate. Assemble the strips in an empty plate holder and wash each well 3 times with 300 µL of diluted Wash Buffer per wash. Decant Wash Buffer and remove the residual amount from all wells 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 automated machine is used for assay, follow the manufacturer's instructions for all washing steps described in this protocol.

9. Add 10 µL Assay Buffer to Background wells and 5 µL Assay Buffer to unknown sample wells.

10. Add 10 µL PIIANP Standards in the order of ascending concentration to the appropriate wells.
11. Add 10 µL QC1 and 10 µL QC2 to the appropriate wells.
12. Add 5 µL of the unknown samples in duplicate to the remaining wells.
13. Add 25 µL Biotin-labeled PIIANP to all wells.
14. Transfer anti-PIIANP Detection Antibody solution to a reagent reservoir and add 50 µL of this solution to each well with a multi-channel pipette. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
15. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
16. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.
17. 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 micro-titer plate shaker.
18. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
19. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.
20. Add 100 µL of Substrate Solution to each well, cover plate with sealer and shake in the plate shaker for approximately 5 to 40 minutes. (A longer development time may be needed if using a plate washer). Blue color should be formed in wells of PIIANP standards with intensity inversely proportional to increasing concentrations of PIIANP.

    Note: One can monitor color development using 370 nm filter, if available on the spectrophotometer. When the absorbance is between 1.2 and 1.8 at 370 nm, the stop solution can be added to terminate the color development.
21. Carefully 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 into 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.

Assay Procedure for Human PIIANP ELISA Kit

	Step 1	Step 2	Step 3	Step 4-6	Step 7	Step 8	Step 9-10	Step 11	Step 12-13	Step 14			
Well #	Dilute both bottles of 10X Wash Buffer with 900 mL Deionized Water.	Wash desired number of strips 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	Assay Buffer	Standards/ Controls/ Samples	Biotin-labeled PIIANP	PIIANP Detection Antibody	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer.	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 3X with 300 µL Wash Buffer.	Substrate	Seal, Agitate, Incubate 5-40 minutes at Room Temperature.	Stop Solution	Shake by hand. Read Absorbance at 450 nm and 590 nm within 5 minutes.
A1, B1			10 µL	-	25 µL	50 µL		100 µL		100 µL		100 µL	
C1, D1			-	10 µL of Tube 6									
E1, F1			-	10 µL of Tube 5									
G1, H1			-	10 µL of Tube 4									
A1, B2			-	10 µL of Tube 3									
C2, D2			-	10 µL of Tube 2									
E2, F2			-	10 µL of Tube 1									
G2, H2			-	10 µL of Reconstituted Standard									
A3, B3			-	10 µL of QC 1									
C3, D3			-	10 µL of QC 2									
E3, F3			5 µL	5 µL of Sample									
G3, H3 ↓			5 µL	5 µL of Sample									

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Microtiter Plate Arrangement

Human PIIANP ELISA

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

Calculations

Graph a reference curve by plotting the absorbance unit of 450 nm, less unit at 590 nm, on the Y-axis against the concentrations of PIIANP standard on the X-axis. 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.

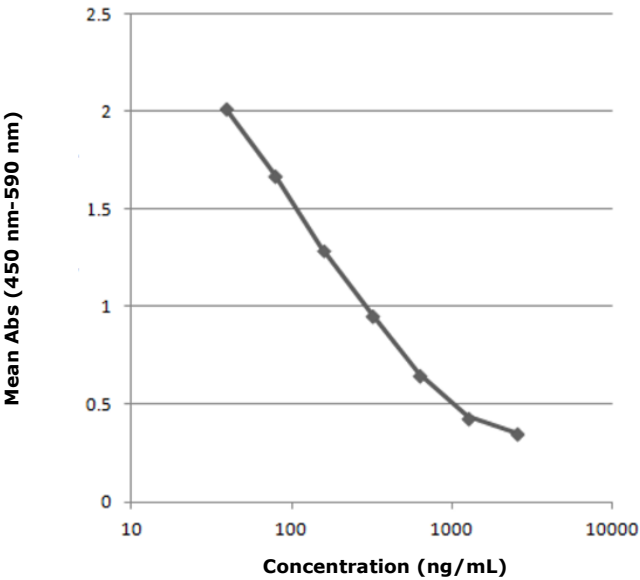
Note: Multiply results for unknown samples by 2 to obtain final PIIANP concentration.

Interpretation

- 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.
- If the difference between duplicate results of a sample is > 15% CV, repeat the sample.
- The limit of sensitivity of this assay is 39 ng/mL.
- Any sample result greater than the last standard concentration should be repeated by diluting the sample at an appropriate dilution in Assay Buffer as diluent immediately prior to setting up the assay.

Graph of Typical Reference Curve

Type IIA Collagen N-Propeptide (PIIANP) ELISA



Typical Standard Curve is for demonstration only. Actual curve from the assay should be used for calculating unknown sample concentrations.

Sensitivity

The lowest level of PIIANP that can be detected by this assay is 39 ng/mL when using a 10 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 Type IIA Collagen N-Propeptide	100%
Human Type I Collagen	0%
Human Type II Collagen	0%
Human Type III Collagen	0%

Precision

Intra-Assay Variation

Sample #	Mean PIIANP (ng/mL)	Assay Variation (%CV)	
		Intra-assay	Inter-assay
1	645	6.60%	7.77%
2	1183	3.43%	7.44%
3	1697	3.37%	4.78%

The assay variations of Human PIIANP ELISA kit were studied on three human serum samples with varying concentrations of spiked analyte. The intra-assay variations are calculated from six singlicate determinations in an assay. The inter-assay variations are calculated from results of 3 separate assays with 6 singlicate determinations in each assay.

Spike Recovery of PIIANP in Human Serum

Serum Sample #	PIIANP		
	PIIANP Added (ng/mL)	Observed (ng/mL)	Recovery (%) of Spiked PIIANP
Human Serum 1	0	461	-
	1000	1478	101.7%
	500	1065	120.8%
	250	789	131.4%
Human Serum 2	0	612	-
	1000	1658	104.5%
	500	1253	128.0%
	250	883	108.4%
Human Serum 3	0	469	-
	1000	1491	102.2%
	500	1063	118.7%
	250	726	102.7%

PIIANP at indicated levels was added to three separate human serum samples and the resulting PIIANP content of each sample was assayed by ELISA.

The % of recovery = [(observed PIIANP level after spike – observed PIIANP level before spike) / spiked level of PIIANP] x 100%.

Linearity of Serum Dilution

Sample	Dilution Factor	PIIANP Level		
		Observed (ng/mL)	Expected (ng/mL)	% of Expected
Human Serum 1	1:1	1690	1690	100.0%
	1:1.33	1138		89.6%
	1:2	753		89.1%
Human Serum 2	1:1	1199	1199	100.0%
	1:1.33	789		87.6%
	1:2	660		110.1%
Human Serum 3	1:1	1070	1070	100.0%
	1:1.33	713		88.6%
	1:2	583		108.9%
Human Serum 4	1:1	964	964	100.0%
	1:1.33	754		104.0%
	1:2	555		115.2%

Four separate human serum samples are diluted each with assay buffer to various degrees as indicated and assayed for PIIANP levels. Measured PIIANP levels are reported as observed PIIANP level.

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 absorbance reading of the blank wells (maximum OD) rise beyond the limit of your microtiter reader's capacity. Adjust the length of substrate incubation time accordingly.

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
Microtiter Plate	EP53
10X HRP Wash Buffer Concentrate	EWB-HRP
PIIANP Standard	E8053-K
Quality Controls 1 and 2	E6053-K
Biotin-labeled PIIANP	EBT53
Assay Buffer	EABPT
Anti-PIIANP Detection Antibody	E1053
Enzyme Solution	EHRP-53
Substrate	ESS-TMB2
Stop Solution	ET-TMB

References

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