DRG® Human PIIANP ELISA (EIA-4446)

Revised 29 Dec. 2010 rm (Vers. 2.1) RUO

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

1 PRINCIPLES OF PROCEDURE

This assay is a competitive ELISA based, sequentially, on: 1) 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, 2) after washing, binding of horseradish peroxidase to the immobilized biotinylated PIIANP, 3) wash away of free enzyme conjugates, and 4) 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 590nm, 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.

2 REAGENTS SUPPLIED

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

A. **Microtiter Plate**
A 96-well microtiter plate containing twelve 8-well columns coated with pre-titered anchor antibodies Quantity: 1 plate. Preparation: Ready to use.
NOTE: Unused strips should be resealed in the foil pouch with the desiccant provided.

B. **Adhesive Plate Sealer**
Quantity: 2 sheets Preparation: Ready to use.

C. **Anti-PIIANP Antibody**
Pre-titered anti-PIIANP antiserum
Quantity: 6 mL Preparation: Ready to use.

D. **10X HRP Wash Buffer Concentrate**
10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
Quantity: Two bottles containing 50 mL each
Preparation: Dilute 1:10 with distilled or de-ionized water.

E. **PIIANP Standard**
Lyophilized PIIANP in buffer
Quantity: 1 vial Preparation: Reconstitute with 1.0 mL of deionized H_2O.
The actual concentration of PIIANP present in the vial will be lot dependent. Please refer to the analysis sheet for exact PIIANP concentration present in a specific lot.

F. **Quality Controls 1 and 2** (Lyophilized)
One vial each, containing PIIANP at two different levels.
Preparation: Reconstitute with 0.2 mL of deionized H_20.

G. **Biotin Labeled PIIANP**
Lyophilized biotinylated PIIANP in buffer
Quantity: 1 vial Preparation: Reconstitute with 5.0 mL of Assay Buffer.

H. **Assay Buffer**
0.01 M phosphate buffer, pH 7.4, containing 0.1% BSA, 0.08% sodium azide, 0.025% Tween 20.
Quantity: 25 mL/vial Preparation: Ready to use.

I. **Enzyme Solution**
Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.
Quantity: 12 mL/vial Preparation: Ready to use.

J. **Substrate (Light Sensitive:** avoid unnecessary exposure to light)
3, 3',5,5'-tetramethylbenzidine in buffer.
Quantity: 12 mL/vial Preparation: Ready to use.

K. **Stop Solution (Caution:** Corrosive Solution)
0.3 M HCl
Quantity: 12 mL/vial Preparation: Ready to use.

3 STORAGE AND STABILITY

All components of the kit can be stored up to two weeks at 2-8°C.

See individual vials for long term storage recommendations. Avoid repeated freeze and thaw cycles. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers. Unused microtiter strips should be resealed in the foil pouch with the desiccant provided.

4 REAGENT PRECAUTIONS

A. Sodium Azide

Sodium azide has been added to certain reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal, flush with large volume of water to prevent azide build up.

B. 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.

5 MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and pipette tips: 5- 20 µl, 20 - 100 µl, 1000 - 5000 µl
2. Multi-channel pipettes and pipette tips: 0 - 50 µl and 50 - 300 µl
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. De-ionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth

6 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 cycles.
5. Avoid using samples with gross hemolysis or lipemia.

7 REAGENT PREPARATION

1) Preparation of Biotin-labeled PIIANP

Rehydrate the provided vial of Biotin-labeled PIIANP with 5.0 mL of deionized or distilled water. 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 -20ºC 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.

2) PIIANP Standard Preparation

1. 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.

2. Label six 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 50µL of tube 1 to tube 2, mix well and transfer 50µL of tube 2 to tube 3, mix well and transfer 50µL of tube 3 to tube 4, mix well and transfer 50µL of tube 4 to tube 5, mix well and transfer 50µL of tube 5 to tube 6, and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standards before dispensing. Unused portions of the reconstituted last standard should be aliquotted 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

3) PIIANP Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using an Eppendorf 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.

8 ASSAY PROCEDURE

Pre-warm all reagents to room temperature immediately 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.
2. 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 ml 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.**
3. Add 10 µl Assay Buffer to the Background wells and 5 µl Assay Buffer to unknown sample wells.
4. Add 10 µl PIIANP Standards in the order of ascending concentration to the appropriate wells.
5. Add 10 µl QC1 and 10 µl QC2 to the appropriate wells.

6. Add 5 µl of the unknown samples in duplicate to the remaining wells.
7. Add 25µl Biotin-labeled PIIANP to all wells.
8. 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.
9. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
10. Wash wells 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.
11. 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.
12. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
13. Wash wells 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.
14. Add 100 µl of Substrate Solution to each well, cover plate with sealer and shake in the plate shaker for **approximately** 20 to 40 minutes. 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.
15. 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. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well. Record the difference of absorbance units.

Revised 29 Dec. 2010 rm (Vers. 2.1) **RUO**

Assay Procedure for Human PIIANP Elisa kit

	Well #	Step 1	Step 2	Step 3	Step 4-6	Step 7	Step 8	Step 9-10	Step 11	Step 12-13	Step 14	
	A1, B1	Dilute both bottles of 10X Wash Buffer with 900mL Deionized Water.	Wash desired number of strips 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	Assay Buffer 10 µL	Standards/ Controls/ Samples ------	Biotin-labeled PIIANP 25 µL	PIIANP Detection Antibody 50 µL	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer	Enzyme Solution 100 µL	Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 3X with 300 µL Wash Buffer	Substrate 100 µL	Stop Solution 100 µL
	C1, D1			------	10 µL of Tube 6							
	E1, F1			------	10 µL of Tube 5							
	G1, H1			------	10 µL of Tube 4							
	A2, 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							

Shake by hand. Read Absorbance at 450 nm and 590 nm within 5 minutes.

9 MICROTITER PLATE ARRANGEMENT

HUMAN PIIANP ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3	QC1									
B	Blank	Tube 3	QC1									
C	Tube 6	Tube 2	QC2									
D	Tube 6	Tube 2	QC2									
E	Tube 5	Tube 1	Sample 1									
F	Tube 5	Tube 1	Sample 1									
G	Tube 4	Reconstituted Standard	Sample 2									
H	Tube 4	Reconstituted Standard	Sample 2									

10 CALCULATIONS

Graph a reference curve by plotting the absorbance unit of 450nm, less unit at 590nm, 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: Multiple results for unknown samples by 2 to obtain final PIIANP concentration.

11 REFERENCES

1. Rousseau JC, Zhu Y, Miossec P, Vignon E, Sandee LJ, Garnero P, Delmas PD. Serum levels of type IIA procollagen amino terminal propeptide (PIIANP) are decreased in patients with knee osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage. 2004 Jun;12(6):440-7.
2. Gouttenoire J, Valcourt U, Ronziere MC, Aubert-Foucher E, Mallein-Gerin F, Herbage D. Modulation of collagen synthesis in normal and osteoarthritic cartilage. Biorheology. 2004;41(3-4):535-42.
3. Garnero P, Ayra X, Rousseau JC, Christgau S, Sandell LJ, Dougados M, Delmas PD. Uncoupling of type II coallagen synthesis and degradation predicts progression of joint damage in patients with knee osteoarthritis. Arthritis Rheum. 2002 Oct;46(10):2613-24.
4. Salminen H, Vuorio E, Saamanen AM. Expression of Sox9 and type IIA procollagen during attempted repair of articular cartilage damage in a transgenic mouse model of osteoarthritis. Arthritis Rheum. 2001 Apr;44(4):947-55.
5. Nah HD, Swoboda B, Birk DE, Kirsch T. Type IIA procollagen: expression in developing chicken limb cartilage and human osteoarthritic articular cartilage. Dev. Dyn. 2001 Apr;220(4):307-22.
6. Aigner T, Zhu Y, Chansky HH, Matsen FA 3rd, Maloney WJ, Sandell L. Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. Arthritis Rheum. 1999 Jul;42(7):1443-50.

7. Zhu Y, Aganesian A, Keene DR, Sandell LJ. Type IIA procollagen containing the cysteine-rich amino propeptide is deposited in the extracellular matrix of prechondrogenic tissue and binds to TGF-beta1 and BMP-2. J Cell Biol. 1999 Mar 8;144(5):1069-80.
8. Oganesian A, Zhu Y, Sandell LJ. Type IIA procollagen amino propeptide is localized in human embryonic tissues. J Histochem Cytochem. 1997 Nov;45(11):1469-80.
9. Sandell LJ, Morris N, Robbison JR, Goldring MB. Alternatively spliced type II procollagen mRNAs define distinct populations of cells curing vertebral development: differential expression of the amino-propeptide. J Cell Biol. 1991 Sep;114(6):1307-19.

Version 02/26/09 ~ rm