Rat/Mouse PINP EIA

For Research Use Only Instructions For Use

Product Name	Rat/Mouse PINP EIA	REF	AC-33F1
Abbreviated Product Name	Rat/Mouse PINP		

1. Intended Use

Not for use in clinical or diagnostic procedures.

The Rat/Mouse PINP EIA assay is a competitive enzyme-linked immunosorbent assay intended for the quantitative determination of N-terminal propeptide of type I procollagen (PINP) in rat or mouse serum or plasma samples. The assay is for research use only.

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2. Summary and Explanation

An important step in the bone formation process is synthesis of type I collagen, which is the major organic component in bone matrix. During collagen synthesis, propeptides are released from both the amino- and carboxyterminal parts of the procollagen molecule[1]. These propeptides are secreted into the blood circulation, and commercially available immunoassays for their measurement from human serum have been developed. Assay for human amino terminal propeptide of type I procollagen (PINP) is probably the most specific and sensitive marker of bone formation[2]. PINP is a particularly useful marker for monitoring the efficacy of osteoporosis therapy with anabolic agents[3-4] , but it is also one of the best bone turnover markers for monitoring the efficacy of antiresorptive therapy[4-5].

The rat/mouse PINP assay is a specific method to determine PINP released during rat and mouse bone collagen synthesis, and it has no cross-reactivity for human PINP. The rat/mouse PINP assay can be used for determining the bone formation rate from rat and mouse serum and plasma samples. Previous studies have demonstrated that PINP secretion is increased after stimulation of mouse osteoblasts by BMP-4 and estrogen in vitro[6]. PINP values are also elevated after treatment with PTH in both young ovariectomized rats and old intact rats[7].

3. Method Description

The Rat/Mouse PINP EIA is a competitive enzyme-linked immunosorbent assay where 50 µL of each calibrators, controls and diluted samples are incubated together with a biotinylated PINP reagent in microtiter wells which are coated with a highly specific polyclonal rabbit anti- PINP antibody at room temperature before aspiration and washing. Enzyme (horseradish peroxidase) labelled avidin is added and binds selectively to complexed biotin and, following a further wash step, colour is developed using a chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures are read in a microtiter plate reader, colour intensity developed being inversely proportional to the concentration of PINP in the original sample.

4. Warnings and Precautions

The Rat/Mouse PINP EIA is for research use only and is not for internal use in humans or animals. This product must be used strictly in accordance with the instructions set out in these Instructions For Use (IFU). Immunodiagnostic Systems Limited (IDS) will not be held responsible for any loss or damage (except as required by statute), howsoever caused, arising out of non-compliance with the instructions provided.

CAUTION: This kit contains material of animal origin. Handle kit reagents as if capable of transmitting an infectious agent. Appropriate precautions and good laboratory practice must be used in the storage, handling and disposal of the kit reagents. Disposal of kit reagents should be in accordance with local regulations.

Reagents containing Sodium Azide

Some reagents in this kit contain sodium azide (NaN_3) <0.1 % (w/w) which may react with lead, copper or brass plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent azide build up.

Calibrators CAL and controls CTRL contain sodium azide (NaN_3) >0.1% (w/w) (<1%).

Classification according to Regulation (EC) CLP:
Skin sensitisation, Category 1

Warning

Contains ProClin 300

Hazard statements:
H317 - May cause an allergic skin reaction.

Precautionary statements
P261 - Avoid breathing dust/fume/gas/mist/vapours/spray.
P280 - Wear protective gloves/protective clothing/eye protection/face protection/hearing protection.
P321 - Specific treatment (see supplemental first aid instruction on this label).
P333+P313 - If skin irritation or rash occurs: Get medical advice/attention.
P362+P364 - Take off contaminated clothing and wash it before reuse.

5. Shelf Life And Storage Of Reagents

Upon receipt, store the kit and components in an **upright** position at 2 - 8 °C. **Do not freeze** the kit or components.
Do not use any kit component beyond their expiry date.

Reagent	**After opening or preparation**
Antibody coated plate	8 weeks Store at 2 – 8°C in foil pouch with desiccant sachet
Biotinylated PINP	8 weeks Store at 2 – 8°C after reconstitution
Wash solution	8 weeks Store at room temperature (18 – 22 °C) after preparation
Calibrator	8 weeks Store at -20°C after reconstitution – stable through 1 freeze/thaw cycle
Controls	8 weeks Store at -20°C after reconstitution – stable through 1 freeze/thaw cycle

Indications of possible deterioration of kit reagents
- The presence of abnormal particulate matter in any of the reagents.
- A decrease in the maximum absorbance.
- A shift in the slope of the curve from its normal position

6. Sample Collection and Storage

The assay should be performed using rat or mouse serum or plasma (heparin or EDTA) samples.

Sample Storage	**Duration**
Room temperature (18 - 22°C)	4 hours
2-8°C	Up to 4 days
Freeze/thaw cycles	Up to 2 cycles

Note:
- Specimens should be separated as soon as possible after collection.
- For long term storage it is recommended to store the samples at -20°C.
- Avoid repeat freeze/thaw cycles for samples.
- The same sample type should be used throughout a study.

7. Materials

Materials Provided

MICROPLAT **Antibody coated plate**
Microwell strips (12x8 wells) pre-coated with polyclonal rabbit anti- PINP antibody, supplied in a plastic frame with desiccant.

PINP BIOTIN **PINP Biotin**
Lyophilised PBS buffer containing PINP labelled with biotin and BSA; 1 vial.

SAMPDIL **Sample Diluent**
Ready to use PBS buffer containing BSA and sodium azide as preservative (0.05 %); 1 vial, 20.0 mL

ENZYMCONJ **Enzyme Conjugate**
Ready to use solution containing peroxidase conjugated to avidin; provided in a buffered solution with protein, enzyme stabiliser and preservative; 1 vial, 18.0 mL

WASHBUF 20x **Wash Concentrate**
Concentrated PBS solution with Tween; 1 vial, 50.0 mL

TMB **Substrate Solution**
Ready to use proprietary aqueous formulation of tetramethylbenzidine (TMB) and hydrogen peroxide; 1 vial, 24.0 mL.
Please note that the chromogenic substrate might appear slightly blueish.

HCL **Stop Solution**
Ready to use solution of 0.5M hydrochloric acid; 1 vial, 13.0 mL.

CAL 0 – 5 **Calibrators**
Lyophilised buffer containing rat/mouse PINP with mouse serum, goat serum and BSA, and <1.0 % sodium azide as preservative (0.025% once reconstituted); 1 each of 6 concentration levels, 0.5 mL per vial.
The exact value of each calibrator is printed on the QC report.

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CTRL 1 **Control 1**
Lyophilised rat serum in PBS buffer containing BSA and with <1.0 % sodium azide as preservative (0.025% once reconstituted); 1 vial, 0.5 mL per vial.
The established range for the control is printed on the QC report.

CTRL 2 **Control 2**
Lyophilised mouse serum in PBS buffer containing BSA and with <1.0 % sodium azide as preservative (0.025% once reconstituted); 1 vial, 0.5 mL per vial.
The established range for the control is printed on the QC report.

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Adhesive Plate Sealer 8 per kit.

Documentation Instructions for Use and QC report.

Materials Required But Not Provided

- Containers for preparing the PINP Biotin and Wash solution
- Precision pipetting devices to deliver 5 – 50 µL
- Distilled or deionised water
- Precision 8 or 12 channel multipipette to deliver 100 µL
- Microplate shaker
- Automatic microplate washer (optional)
- Photometric microplate reader and data analysis equipment

8. Preparation Of Reagents

Allow all reagents to come to room temperature for a minimum of 60 minutes before use. Do not interchange kit components from different lots.

Wash Solution

Add the contents of the Wash Concentrate WASHBUF 20x to 950 mL of distilled or deionised water and mix. Store at room temperature.

PINP Biotin PINP BIOTIN
PINP Biotin is supplied lyophilised. Add 8 mL of Sample Diluent SAMPDIL to the bottle of lyophilised PINP Biotin PINP BIOTIN. Replace the stopper and allow to stand for 5 – 10 minutes at room temperature (18 - 22°C)
- Invert several times to ensure complete reconstitution.

Calibrators CAL and Control CTRL: Calibrators CAL and Control CTRL are supplied lyophilised. Reconstitute with 0.5 mL of distilled or deionised water, replace stopper and allow to stand for 5 – 10 minutes at room temperature (18 - 22°C). Invert several times to ensure complete reconstitution.

If Calibrators or Controls are to be used more than once they must be frozen (-20°C) as soon as possible after reconstitution. When reusing Calibrators and Controls, thaw at room temperature, mix and use within 15 minutes of completion of thaw.

All other reagents are supplied ready for use and should be mixed by repeated inversion before use.

N.B. To avoid potential microbial and / or chemical contamination, unused reagents should never be returned into the original vials.

9. Assay Procedure

Prepare reagents as described in § 8. Preparation of Reagents. Mix all reagents and samples before use (avoid formation of foam).
NOTE: To ensure consistent results between runs, between operators, and to minimise any drift effect; strictly adhere to the following procedure:

a. Bring all reagents to room temperature (18 – 22 ºC) prior to use – this will take approximately 60 minutes.
b. Seal the plate during incubations using the plate sealers which are supplied with the assay kit.
c. Do not stack plates during incubation to ensure a consistent temperature for all plates.
d. Do not under or over-fill the assay wells during the washing steps.
e. Calibrators and control must be run in columns 1 and 2 only.
f. Add conjugate within 5 minutes of washing.
g. Add substrate within 5 minutes of washing; stagger stop times as needed.
h. Add reagents in the same sequence each time to reduce time deviation between reactions.

Do not pipette directly from the vial containing TMB substrate. The required volume should first be transferred to a clean container. Solution remaining in the container should be discarded following use and NOT returned to the stock vial SUBS TMB

Determine the number of strips needed for the assay; it is recommended to test all samples in duplicate. In addition, for each run a total of 16 wells are needed for the standards and controls. Place the appropriate number of strips in the plastic frame. Store any unused strips in the tightly closed foil bag with desiccant capsules.

1. Pipette 50 µL of each standard CAL 0 – 5 and control CTRL 1 – 2 to the appropriate wells on the Antibody Coated Plate MICROPLAT
2. Pipette 5 µL of sample and 45 µL Sample Diluent SAMPDIL appropriate wells on the Antibody Coated Plate MICROPLAT
3. Pipette 50 µL of Biotinylated PINP reagent PINP BIOTIN into each well.
4. Cover the plate with an adhesive plate seal.
5. Incubate at room temperature (18 - 22°C) for 1 hour on a microtiter plate mixer (500 - 700 rpm).
6. Wash all wells 3 times with wash solution WASHBUF SOLN
   a. Automatic plate wash — Set plate washer to dispense 300 µL of wash solution per well.
      a. Fill and aspirate for 3 cycles.
   b. Manual wash — Decant the contents of the wells by inverting sharply.
      a. Pipette 250 µL of wash solution into each well, decant and repeat 3 times.
      b. Remove excess wash buffer by tapping firmly on absorbent tissue before proceeding.
7. Pipette 150 µL of Enzyme Conjugate ENZYMCONJ into each well.
8. Cover the plate with an adhesive plate seal.
9. Incubate at room temperature (18 - 22°C) for 30 minutes.
10. Repeat wash step 6.
11. Pipette 150 µL of TMB Substrate TMB into each well
    NOTE: do not pipette directly from the vial containing TMB substrate. The required volume should first be transferred to a clean container. Solution remaining in the container should be discarded following use and NOT returned to the stock vial TMB
12. Cover the plate with an adhesive plate seal.
13. Incubate at room temperature (18 - 22°C) for 30 minutes in the dark.
14. Pipette 50 µL of Stop Solution HCL into each well.
15. Measure absorbance at 450 nm with reference at 650 nm using a microplate reader within 30 minutes of stopping the reaction.

N.B. Microplate readers measure vertically; when pipetting, do not touch the bottom of the wells.

Automated Platforms
The RatMouse PINP EIA kit was designed and developed to be performed manually using the protocol described above. The protocol is not necessarily applicable to automated platforms.
If automated platforms are used it is the responsibility of the user to ensure the kit has been appropriately tested. To improve the performance of the kit on automated platforms, it is recommended to increase the number of wash cycles at each wash step.

10. Calculation of Results

A variety of data reduction software packages are available, which may be employed to generate the mean calibration curve and to calculate the mean concentrations of unknown samples and controls. A 4 parameter logistic (4PL) curve fit, **including Calibrator 0 is required.**

Alternatively, a calibration curve may be prepared on semi-log graph paper by plotting mean absorbance on the Y-axis against concentration of PINP on the X-axis. Calibrator 0 should be included in the calibration curve. Read the mean absorbance value of each unknown sample off the curve.

NOTE: To obtain the concentration of PINP in each sample, multiply the value read from the curve by the dilution factor used (x10).

11. Quality Control

Good Laboratory Practice (GLP) requires the use of quality control specimens in each series of assays in order to check the performance of the assay. Controls should be treated as unknown samples, and the results analysed with appropriate statistical methods.

The two kit controls provided in the kit should be tested as unknowns and are intended to assist in assessing the validity of results obtained with each assay plate.

IDS recommends the users to maintain graphic records of the control values generated with each assay run, including the running means, SDs and %CVs. This information will facilitate the controls trending analysis relating to the performance of current and historical control lots relative to the supplied Quality Control data. The trending will assist in the identification of assays which give control values significantly different from their average range.

In order to properly evaluate the performance of the assay, IDS recommends that all laboratories include in each assay appropriately aliquoted and stored in-house pools in addition to the controls provided with the kit. Quality Control charts should be maintained to follow the assay performance.

When interpreting control data, users should note that this product was designed and developed as a manual product. The range stated on the QC certificate should be appropriate for assays that are performed manually and with strict adherence to the Assay Procedure described above. It is recognised by Quality Control professionals, that as a result of differences in conditions and practices, there will always be variability in the mean values and precision of control measurements between different laboratories[8].

12. Limitations of Use

- The following substances do not interfere in the Rat/Mouse PINP EIA when the concentrations presented in the following table are below the stated threshold.

Potentially Interfering Agent	Threshold Concentration
Conjugated Bilirubin	Up to 30 mg/dL
Haemoglobin	Up to 500 mg/dL
Triacylglycerol (TAG)	Up to 600 mg/dL

13. Representative Performance Data

Representative performance data are shown which are calculated based on a limited level of testing and are provided **for guidance only**. Results obtained at individual laboratories may vary.

13.1 Sensitivity

The analytical sensitivity, the concentration corresponding to the Mean-2SD of 20 replicates of the zero calibrator, is 0.33ng/mL (3.3 ng/mL sample equivalent, after x10 dilution correcting factor).

13.2 **Precision**

Intra-assay precision was evaluated by running a total of 6 samples in 20 replicates using 1 lot of reagent.

Sample	Sample Equivalent Mean conc. (ng/mL)	Mean conc. (ng/mL)	SD (ng/mL)	CV%
1	32.4	3.2	0.3	10.1
2	124.7	12.5	0.9	7.3
3	183.2	18.3	1.4	7.8
4	274.6	27.5	3.8	13.9
5	281.9	28.2	2.3	8.1
6	291.9	29.2	3.5	11.8

Inter-assay precision was evaluated by running a total of 2 kit controls and 3 pools using 1 lot of reagent in duplicate, across 37 assay runs.

Sample	Sample Equivalent Mean conc. (ng/mL)	Mean conc. (ng/mL)	SD (ng/mL)	CV%
1	50.9	5.1	0.4	8.4
2	418.4	41.8	3.9	9.3
3	55.0	5.5	0.6	10.6
4	252.4	25.2	2.9	11.3
5	535.5	53.6	4.8	9.0

13.3 Linearity

Linearity was evaluated by diluting high and low samples prior to assay, with each dilution being assayed in duplicate. The resulting mean observed concentrations were compared with the predicted expected concentrations. The mean value for %observed/expected for each sample type (7 dilutions of 2 samples per sample type) was determined as:

Rat		Mouse serum
Serum	Plasma	
95%	111%	102%

Linear Regression analysis of all samples combined with expected (x-axis) and observed (y-axis) values:
Observed = 1.05 x Expected – 4.9ng/mL r = 0.98.

13.4 Method Comparison

The Rat/Mouse PINP EIA, using the new formulation of rabbit anti-PINP antibody was compared against the previous formulation of the assay. A total of 86 neat samples selected to include the various sample types was assayed by each method. Passing-Bablok regression analysis was performed on the comparative data:

n	Slope	95% CI	Intercept (ng/mL)	95% CI	Correlation Coefficient (r)
86	1.08	1.00 to 1.17	-4.0	-15.8 to 4.5	0.95

13.5 Specificity

The specificity was assessed with the following cross-reactants.

Cross Reactant	% Cross-Reactivity
Mouse PINP	100 %
Rat PINP	100 %
Human PINP (2,00 ng/mL)	Not detectable
Human PIIINP (5000 ng/mL)	Not detectable
Mouse/Rat PIIINP (5000 ng/mL)	Not detectable

14. Symbols used

REF Catalogue Number

Manufacturer

15. Bibliography

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Assay Procedure