

# METRA™ Serum PYD EIA kit

96 Assays for Pyridinoline Crosslinks in Serum

For Research Use Only. Not for use in diagnostic procedures.

Store at 2-8°C

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**QUIDEL®**

Read the entire product insert thoroughly before beginning the assay. The Serum Pyd kit should be stored at 2-8°C until use.

## QUICK GUIDE TO ASSAY STEPS

1. Add 50µL Reagent 1 to all wells using a multichannel pipette.
2. Add 25µL diluted Standards, diluted Controls, and **undiluted**, filtered samples.
3. Add 75µL cold Pyridinoline antibody.
4. Incubate overnight at 2-8°C in the dark.
5. Wash 3 times with 1X Wash Buffer.
6. Add 150µL room temperature Enzyme Conjugate.
7. Incubate 60 ± 5 minutes at room temperature.
8. Wash 3 times with 1X Wash Buffer.
9. Add 150µL room temperature Working Substrate Solution.
10. Incubate 40 ± 5 minutes at room temperature.
11. Add 100µL Stop Solution and read OD at 405nm.

## INTENDED USE

The Serum Pyd assay provides a quantitative measure of the excretion of pyridinoline crosslinks in serum.

## SUMMARY AND EXPLANATION

Structural collagens such as types I and II that are present in bone and cartilage, respectively, are crosslinked both within their  $\alpha$ -chains and between adjacent molecules to provide rigidity and strength to the resulting collagen fibril<sup>1-3</sup>. In bone and cartilage, pyridinoline (Pyd), a trifunctional pyridinium crosslink, forms between specific hydroxylysine residues within the telopeptide regions of one collagen molecule and within the helical region of an adjacent collagen molecule<sup>3</sup>. When bone or cartilage collagen is degraded, Pyd is released into circulation and excreted in the urine. Deoxypyridinoline (Dpd), a pyridinium crosslink differing from Pyd only by the absence of an hydroxyl group, is essentially bone specific<sup>1,2,4</sup>. The ratio of Pyd:Dpd in bone of approximately 4:1 is closely concordant with the ratio observed in urine of healthy individuals and those with metabolic bone diseases or osteoarthritis in spite of a much higher Pyd:Dpd ratio in nonbone tissues<sup>2,4</sup>. This suggests that both crosslinks reflect bone resorption under these conditions. In rheumatoid arthritis, destruction of cartilage and other collagen-containing joint tissue may contribute to increased levels of Pyd<sup>2</sup>.

In humans, the total pool of urinary Pyd is approximately 45% free while the remaining fraction is bound to oligopeptides ranging from small linear peptides to very large crosslinked structures in excess of 10,000 Da<sup>4,7</sup>. The proportion of free to total crosslinks appears to be constant in healthy individuals and those with metabolic bone diseases or arthritis conditions<sup>4,6,8</sup>, thus providing the rationale for measuring free Pyd. Improvements in immunoassay sensitivity have resulted in the ability to measure free Pyd levels in serum, thus permitting a novel method for researching bone and cartilage collagen degradation<sup>9,10</sup>. Since Pyd is present and excreted in all mammalian species evaluated, including rodents, dogs, sheep, horses, and nonhuman primates, research applicability of a serum Pyd immunoassay extends to animal model studies.

## PRINCIPLE OF THE PROCEDURE

The Serum Pyd assay is a competitive enzyme immunoassay in a microtiter plate format. Pyd in the samples or standards competes with Pyd immobilized on the plate for polyclonal rabbit anti-Pyd antibody. Bound antibody is detected by goat anti-rabbit antibody conjugated to alkaline phosphatase and the reaction is detected with pNPP substrate.

## REAGENTS AND MATERIALS

**Metra Serum Pyd EIA Kit, part number 8019, contains the following:**

<b>Substrate Tablets</b>	<b>Part 0012</b>	<b>3 each</b>
p-Nitrophenyl phosphate (20mg each).		
<b>Pyridinoline Antibody</b>	<b>Part 4543</b>	<b>9 mL</b>
Rabbit anti-Pyd polyclonal antibody containing buffer salts and sodium azide (0.05%) as a preservative.		
<b>Enzyme Conjugate</b>	<b>Part 4544</b>	<b>3 each</b>
Lyophilized goat anti-rabbit antibody conjugated to alkaline phosphatase containing buffer salts and stabilizers.		
<b>Pyridinoline Standard: 120nmol/L</b>	<b>Part 4552</b>	<b>0.3 mL</b>
Pyd purified from human urine in 10mmol/L phosphoric acid containing sodium azide (0.05%) as a preservative.		
<b>Low/High Controls</b>	<b>Parts 4553, 4554</b>	<b>0.3 mL each</b>
Pyd purified from human urine in 10mmol/L phosphoric acid containing sodium azide (0.05%) as a preservative.		
<b>Coated Strips, 1 X 8 wells</b>	<b>Part 4668</b>	<b>1 each</b>
Pyd purified from bovine bone adsorbed onto stripwells.		
<b>Stop Solution</b>	<b>Part 4702</b>	<b>15 mL</b>
1N NaOH		

## REAGENTS AND MATERIALS (CONT.)

<b>10X Wash Buffer</b> Nonionic detergent in a buffered solution containing sodium azide (0.05%) as a preservative.	<b>Part 4703</b>	<b>55 mL</b>
<b>Assay Buffer</b> Nonionic detergent in a buffered solution containing sodium azide (0.05%) as a preservative.	<b>Part 4704</b>	<b>55 mL</b>
<b>Substrate Buffer</b> A diethanolamine and magnesium chloride solution containing sodium azide (0.05%) as a preservative.	<b>Part 4705</b>	<b>10 mL</b>
<b>Reagent 1</b> A glycine solution containing indicator dye and ProClin (0.05%) as a preservative.	<b>Part 4744</b>	<b>6 mL</b>

## WARNINGS

For Research Use Only. Not for use in diagnostic procedures.

- All serum samples should be treated as potentially biohazardous material.
- The standard and controls contain Pyd purified from human urine and should be treated as potentially biohazardous material.
- Standard and controls are in 10mmol/L phosphoric acid. Avoid contact with skin, eyes or clothing. Do not ingest. If contact is made, wash with water. If ingested, call a physician.
- 1N NaOH is poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing. If contact is made, wash with water. If ingested, call a physician.
- Sodium azide is used as a preservative. It may be fatal if swallowed or absorbed through the skin. Do not mix with acids. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with a large volume of water to prevent azide build-up.
- Test kits and components should be disposed of in a manner consistent with relevant regulations.

## PRECAUTIONS

- The Pyridinoline standard, controls, coated strips and serum samples are light sensitive. Avoid prolonged exposure to light, especially direct or indirect sunlight. Store reagents in the dark when not in use. Samples and reagents are not significantly affected by normal, artificial laboratory lighting when handled as directed in the Assay Procedure.
- All reagents supplied should be used as an integral unit prior to the expiration date indicated on the package label.
- Assay reagents should be stored as indicated.
- Do not use Coated Strips if pouch is punctured.
- Samples greater than 12nmol/L should be further diluted in Assay Buffer and retested. Include the dilution factor in the final calculation.
- Each sample should be tested in duplicate.
- A standard curve must be performed with each assay.
- A 4-parameter calibration curve fit must be used for accurate results. Equation:  $y = (A-D)/(1+(x/C)^B)+D$
- This assay may be performed with any validated washing method.

## PRECAUTIONS (CONT.)

- The Certificate of Analysis included in this kit is lot specific and is to be used to verify that the results obtained by your laboratory are similar to those obtained at Quidel. The OD values provided are to be used as a guideline only. The results obtained by your laboratory may differ.

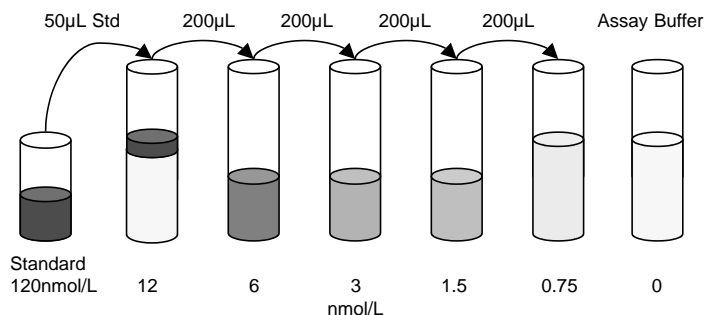
Quality control ranges are provided. The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits. If the control values are NOT within your laboratory's acceptance limits, the assay results should be considered questionable and the samples should be repeated.

- If the OD of the zero standard (Assay Buffer) is less than 0.8, the results should be considered questionable and the samples should be repeated.
- If room temperature cannot be maintained between 20-28°C and an absorbance of > 2.0 is not compatible with your plate reader, monitor the development of substrate in the zero standard wells; stop the reaction when the OD reaches 1.2-1.5; then read the strip(s).
- Use of multichannel pipettes is essential to ensure the timely delivery of reagents. Reagent addition should be uninterrupted.
- For accurate measurement of samples, the addition of samples and standards must be precise. Pipet carefully using only calibrated equipment.

## REAGENT PREPARATION AND STORAGE

- Wash Buffer**  
Prepare required amount of 1X Wash Buffer (see table in Assay Procedure Section) by diluting 10X Wash Buffer 1:10 with deionized water. Store at room temperature (20-28°C). Use 1X Wash Buffer within 24 hours of preparation.
- Enzyme Conjugate**  
Prepare Enzyme Conjugate within 2 hours of use. Reconstitute each required vial of Enzyme Conjugate (see table) with 10mL of 1X Wash Buffer. Store reconstituted Enzyme Conjugate at room temperature (20-28°C) until use.
- Working Substrate Solution**  
The Substrate Buffer must be brought to room temperature (20-28°C) before beginning the assay. (Two hours to overnight recommended.) Prepare Working Substrate Solution within 1 hour of use. Put one Substrate Tablet into each required bottle of room temperature Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.
- Pyd Standard**  
Prepare 12nmol/L Standard Solution by diluting the 120nmol/L Pyd Standard 1:10 with Assay Buffer (50µL Pyd Standard + 450µL Assay Buffer).

Use the 1:10 12nmol/L Standard Solution prepared above to produce the 1:2 dilution series depicted below (200µL Working Standard + 200µL Assay Buffer). Mix each tube thoroughly before the next transfer. The 1:10 Standard Solution serves as the high standard (12nmol/L) and Assay Buffer serves as the zero standard (0nmol/L). A fresh set of standards should be prepared each day.



## REAGENT PREPARATION AND STORAGE (CONT.)

5. Controls  
Dilute Controls 1:10 with Assay Buffer (50µL Control + 450µL Assay Buffer).

**NOTE: Cloudiness, discoloration, or offensive odor may indicate instability or deterioration of kit reagents. If this occurs, the reagent should be discarded.**

## SPECIMEN COLLECTION AND STORAGE

Collect the serum using standard venipuncture technique. Specimens should be processed to avoid hemolysis. Keep the serum sample refrigerated (2-8°C) for storage of less than 4 days, or freeze the sample at  $\leq -20^{\circ}\text{C}$  for longer storage. Do not subject the samples to more than 3 freeze/thaw cycles. Avoid prolonged exposure to light, especially direct sunlight. Samples are not affected by normal, artificial laboratory lighting.

## SAMPLE PREPARATION

Filter serum samples by adding approximately 200µL of each serum to a 30k MWCO Spinfilter (1 basket and 1 receptacle per spinfilter). Centrifuge approximately 30 minutes at 3,000xg to 10,000xg. Filtrate should be colorless. Repeat filtration if filtrate is yellow. Protect filtrate from prolonged (more than 1 hour) exposure to light. Samples should be prepared for same day use.

## SERUM PYD ASSAY PROCEDURE

Metra Serum PYD part number 8019

<b>Kit Contents</b>	<b>Qty/Vol</b>	<b>Part</b>
Substrate Tablets	3 each	0012
Tape Cover	3 each	0047
Pyridinoline Antibody	9mL	4543
Enzyme Conjugate (lyophilized)	3 each	4544
Standard (Pyd 120nmol/L)	0.3mL	4552
Control, Low	0.3mL	4553
Control, High	0.3mL	4554
Coated Strips (12)	1 each	4668
Stop Solution	15mL	4702
10X Wash Buffer	55mL	4703
Assay Buffer	55mL	4704
Substrate Buffer (3)	10mL	4705
Reagent 1	6mL	4744
30k MWCO Spinfilter (packaged separately)	40 each	0590, 0768, or 4935

### Materials Required BUT NOT Provided

Micropipettes to deliver 25-300µL  
Multichannel pipette  
Labware suitable for liquid measurement of 10-300mL  
Deionized or distilled water  
Glass or plastic tubes for dilution of standard and controls  
Centrifuge capable of 3,000xg to 10,000xg and able to accommodate spinfilter tubes  
Plate reader capable of reading at 405nm  
4-parameter calibration curve fitting software

Determine amount of each reagent required for the number of strips to be used.

<b># of Strips</b>	<b>4</b>	<b>6</b>	<b>8</b>	<b>12</b>
<b># of Samples</b> (tested in duplicate)	<b>8</b>	<b>16</b>	<b>24</b>	<b>40</b>
Enzyme conjugate (vial)	1	1	2*	2*
Substrate Buffer (bottle)	1	1	2*	2*
1X Wash Buffer (mL)	100	150	200	300

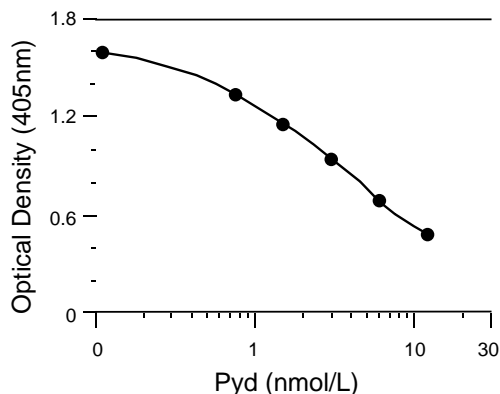
\*When more than one bottle or vial is to be used, combine the contents of each vial, and mix prior to use.

## SERUM PYD ASSAY PROCEDURE (CONT.)

1. Prepare Standards, Controls, samples and reagents as instructed.
2. Allow pouch of Coated Strips to equilibrate to room temperature before opening. Remove Stripwell Frame and the required number of Coated Strips from the pouch (see table). Ensure that the pouch containing any unused strips is completely resealed and contains desiccant.
3. Add 50µL Reagent 1 to each well of the Coated Strips using a multi-channel pipette.
4. Add 25µL diluted Standard, Control, or undiluted filtered sample to each well of the Coated Strips. This step should be completed within 20 minutes.
5. Add 75µL of cold Pyridinoline antibody to each well. Dispense Pyridinoline antibody with sufficient force to ensure adequate mixing. Tap Stripwell Frame several times. Cover plate with Tape Cover provided. Incubate overnight (18-24 hours) at 2-8°C. This incubation should be carried out in the dark.
6. Manually invert/empty strips. Add at least 250µL of prepared 1X Wash Buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash.
7. Add 150µL of room temperature reconstituted Enzyme Conjugate to each well (add 10mL of 1X Wash Buffer to each required vial). Incubate for  $60 \pm 5$  minutes at room temperature (20-28°C).
8. Manually invert/empty strips. Add at least 250µL of 1X Wash Buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash. While the strips are inverted, carefully wipe bottom of strips with a lint-free paper towel to ensure that the bottom of the strips are clean.
9. Add 150µL of Working Substrate Solution to each well. Incubate for  $40 \pm 5$  minutes at room temperature (20-28°C).
10. Add 100µL of Stop Solution to each well to stop the reaction. Add Stop Solution in the same pattern and time intervals as the Substrate Solution addition.
11. Read the Optical Density (OD) at 405nm. Assure that no large bubbles are present in wells and that the bottom of the strips are clean. Strips should be read within 15 minutes of Stop Solution addition.
12. Quantitation software with a 4-parameter calibration curve fitting equation must be used to analyze Serum Pyd assay results.
13. Determine concentration of samples and Controls from the standard curve.
14. Control values should be within the range specified in the Certificate of Analysis supplied with the kit.

### Representative Standard Curve

Standard Pyd levels: 0, 0.75, 1.5, 3, 6, 12 nmol/L



## INTERPRETATION OF RESULTS

Sample results are expressed as nM/L and **do not** need to be corrected for dilution.

## SERUM PYD EXPECTED VALUES

Preliminary Serum Pyd reference ranges have been established for healthy premenopausal females (n=26) and males (n=33) over 25 years of age. For the purposes of establishing reference ranges, normal subjects were defined as:

- Basically healthy, no bone, endocrine or chronic disorders
- Regular menstrual cycles (females)
- Not pregnant or breast feeding (females)
- Not currently taking any medication known to influence bone metabolism (eg. anticonvulsants, bisphosphonates, calcitonin, corticosteroids, GnRH analogs, heparin, hormone replacement therapy, and thyroid medication)

Sex	Age	Mean	SD
Females	25-44	1.55	0.26
Males	25-55	1.59	0.38

## PERFORMANCE CHARACTERISTICS

### Specificity

The Pyridinoline antibody demonstrates selective, high affinity for free pyridinoline and negligible binding to Dpd, and Pyd and Dpd peptides.

	% Reactivity
Free Pyd	100%
Free Dpd	0%
Pyd/Dpd peptides ≥ 1000 MW	< 2.5%
L-Arginine	0.0004%
L-Glutamine	0.000009%

Physiological levels of arginine and glutamine contribute to the measured Pyd value (equivalent to approximately 0.5nmol/L in humans). Physiological levels of other amino acids are undetectable.

### Reactivity to Pyridinoline in Animal Sera

The Pyridinoline antibody demonstrates reactivity to free Pyd from the following animal species: mouse, rat, guinea pig, dog, cow, horse and primate (monkey, baboon).

(Rabbit reacts but is not compatible in this assay due to possible interference of endogenous rabbit antibodies.)

### Sensitivity

The minimum detection limit of the Serum Pyd Assay is 0.4nmol/L, determined by the upper 3 SD limit in a zero standard study.

### Precision

Within-run and between-run precision were determined by assaying four serum samples.

Pyd nmol/L	Within-run <sup>1</sup> CV%	Between-run <sup>2</sup> CV%
1.04	14.8	11.6
2.26	8.3	8.7
4.63	6.3	9.5
8.13	8.0	10.4

<sup>1</sup> 12 runs

<sup>2</sup> 20 runs

## PERFORMANCE CHARACTERISTICS (CONT.)

### Recovery - Linearity

Linearity was determined by serially diluting samples of all listed species and comparing observed values with expected values.

Average recovery was 107%.

Absolute range: 93 - 122%.

### Recovery - Spike Recovery

Spike recovery was determined by adding known quantities of purified Pyd to serum samples of all listed species.

Average recovery was 100%.

Absolute range 91 - 109%.

### Interfering Substances

The following substances were tested at the specified concentrations, and were not found to interfere with the assay.

Substance	Concentration
Bilirubin, conjugated	40mg/dL
Cholesterol	500mg/dL
Hemoglobin	500mg/dL
Protein, Total	12g/dL
Protein, Albumin	6g/dL
Protein, $\gamma$ -Globulin	6g/dL
Triglycerides (lipemia)	3000mg/dL

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