MA-0101

Hydroxyproline Assay

(100 wells, Colorimetric, OD 560 nm, Store at +4°C)

Background Information:

Hydroxyproline, (MW 131.13), a nonessential amino acid is found primarily in collagen and elastin in animals. Hydroxyproline-rich proteins are also found in the cell walls of plants where it is used as a glycan attachment point. Other instances of hydroxyproline in proteins are known but relatively rare (HIF-1, conotoxin, etc.). Hydroxyproline is formed in an ascorbate dependent process by prolyl hydroxylase after synthesis of the peptide chain. It functions in collagen by strengthening mechanical integrity. Collagen formation and turnover in disease states (liver fibrosis, Paget's, bone metastases) can be assessed through measurement of hydrolysates or serum and urine hydroxyproline levels. AkrivisBio's Hydroxyproline Assay is designed to measure hydroxyproline primarily in protein and tissue hydrolysates. Serum and urine can be used as samples after appropriate pretreatment. AkrivisBio provides a simple method of measuring hydroxyproline giving a chromogen which can be measured between 550-565 nm, useful over the range of 1-20 µg of collagen or about 0.1-2 µg of hydroxyproline.

Assay Principle:

1 - Samples are thoroughly hydrolyzed in acid
2 - Hydroxyproline is oxidized by Chloramine T to a pyrrole
3 – The pyrrole is subsequently reacted with Ehrlich's Reagent to form a chromophore.

Assay Components:

Oxidation Buffer	10 ml	WM	MA-0101A
Chloramine T Concentrate	0.6 ml	Red	MA-0101B
Acid/Isopropanol Solution	5 ml	NM	MA-0101C
DMAB Concentrate	5 ml	Amber	MA-0101D
Hydroxyproline Standard (1 mg/ml)	0.1 ml	Yellow	MA-0101E
Microplate Sealing Film	1 ea	-------	MA-0101F

User Supplied Reagents and Equipment:

12 M HCl
96-well clear plate with flat bottom
Oven or hot plate
Polypropylene Vials

Storage and Handling:

Store kit at +4°C. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **Chloramine T Reagent:** For each well, add 6 µl of Chloramine T Concentrate to 94 µl of Oxidation Buffer and mix well.
- **DMAB Reagent**: For each well, add 50 µl of DMAB Concentrate to 50 µl of Acid/Isopropanol Solution and mix well.

Note: Use diluted reagents within 2-3 hours for best results.

Assay Protocol:

1. Sample Preparation:

Homogenize samples using 100 µl DI H_2O/10 mg of tissue. Add an equal volume of concentrated HCl (~12N, not provided) in a pressure-capable vial (such as Nalgene Micro Vial), mix well and hydrolyze at 120°C for 3 hours. Treat urine samples with an equal volume of concentrated HCl and hydrolyze the same way. Urine samples should then be treated with activated charcoal (1 mg / 50 µl of urine/HCl mix). Centrifuge for 3 min. to remove activated charcoal. Transfer 10 µl of each hydrolyzed sample to a 96-well plate and evaporate to dryness under heat and/or vacuum.

Note: Endogenous compounds may interfere with the reaction. To ensure accurate determination of Hydroxyproline in the test samples, we recommend spiking samples with a known amount of Standard (0.4 µg).

2. Standard Curve:

Dilute the Hydroxyproline Standard to 40 µg/ml by adding 10 µl of the 1 mg/ml Standard to 240 µl of DI H_2O, mix well. Add 0, 5, 10, 15, 20, 25 µl into a series of wells in duplicate. This will give a standard curve of 0 - 0.2 - 0.4 - 0.6 - 0.8 - 1 µg/well.

3. Oxidation Reaction:

Add 100 µl of the **Chloramine T reagent** to each sample and standard. Allow oxidation to occur at RT for 5 min.

4. Color Development Reaction:

Add 100 µl of the **DMAB Reagent** to each well, apply plate sealer and allow full color development to occur for 30-40 minutes at 60°C.

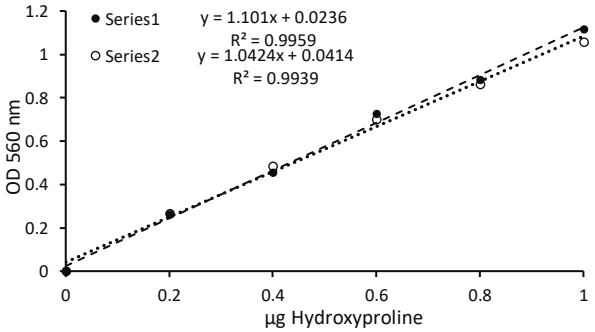
Note: Chloramine T reagent and DMAB Reagent have very different densities and do not mix easily. Pipette each well up and down 2-3 times to mix before sealing plate. Color development reaches a maximum, around 30 minutes. It will fade if you cook it longer. Color persists at room temperature.

5. Measurement:

Measure absorbance at 560 nm.

6. Typical Result:

µg Standard	Standard Raw Values OD		Background Corrected Values OD	
0	0.0371	0.0402	0	0
0.2	0.301	0.3080	0.2639	0.2678
0.4	0.4923	0.5249	0.4552	0.4847
0.6	0.7647	0.7407	0.7276	0.7005
0.8	0.9193	0.9039	0.8822	0.8637
1.0	1.1531	1.0988	1.1160	1.0586



7. Calculation:

Subtract the 0 µg Hydroxyproline Standard OD from all readings. Plot the Standard curve. The slope defines the system sensitivity. Divide the background corrected sample readings by the slope of the standard curve to determine the amount of hydroxyproline in the test wells. Extrapolate back to amount in the original samples by:

A. Divide the hydroxyproline amount in the well by the volume of sample in µl added to the well = µg hydroxyproline/µl of sample
B. Multiply µg hydroxyproline/µl of sample by total volume of sample in 1. above = total hydroxyproline in sample
C. Multiply total hydroxyproline in sample by any dilution factor incurred during processing.
D. Divide total hydroxyproline in sample by mg tissue or volume of liquid sample before processing = µg hydroxyproline / mg tissue, etc.

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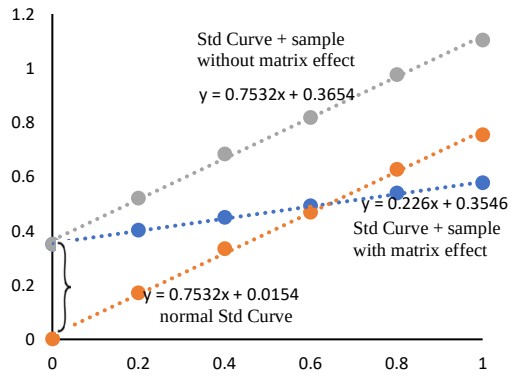
Note: Many metabolites and other chemicals can interfere significantly with a variety of reaction chemistries. For more precise determinations, perform a standard curve in the absence and presence of a constant amount of sample. It's not necessary to run all six standards; use a minimum of 3 (0 – 10 – 20 µl) to verify that the OD response is linear with amount of analyte. The slopes of the two std curves should be the same. The OD offset between them is attributable to the amount of analyte (hydroxyproline in the current case) in the sample. If the two slopes are different, there is a matrix effect influencing the reaction chemistry. In that case determine the OD difference between the 0 standards and apply the slope of the standard curve run in the presence of sample to it.

Example of a sample with a large matrix effect:



The difference between 0 Standards is 0.35. Slope in the presence of sample is 0.226 OD/µg. Amount of hydroxyproline in sample with matrix effect = 0.35 OD / 0.226 OD/ µg = 1.55 µg

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