Revised Protocol

Product Manual

Hydroxyproline Assay Kit (Perchlorate-Free)

Catalog Number

STA-675 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Hydroxyproline is an amino acid that is synthesized from the irreversible post-translational hydroxylation of proline by prolyl hydroxylase. Hydroxyproline is found almost exclusively in the protein collagen, in the Y position of the repeating tripeptide Gly-X-Y. By allowing sharp twisting of the collagen helix, hydroxyproline helps to stabilize the structure of collagen. In addition to collagen, hydroxylation of proline has been observed on the transcription factor Hypoxia Inducible Factor (HIF-1). Under normal oxygen conditions the protein EGLN1 hydroxylates HIF-1 alpha at proline 564, allowing ubiquitylation by the von Hippel-Lindau tumor suppressor (pVHL) and causing the targeting of HIF-1 for degradation by the proteasome.

Since hydroxyproline has been found on so few proteins other than collagen, measurement of hydroxyproline has been used as a marker to quantify levels of collagen and/or gelatin (partial hydrolysis of collagen resulting in a mixture of protein and peptides). It is estimated that hydroxyproline makes up 13.5% of collagen. In addition, hydroxyproline measurement has been used to identify certain diseases that involve breakdown of collagen. For example, increased levels of collagen have been measured in serum of Paget's Bone Disease. In addition, increased hydroxyproline levels have been correlated with prostatic carcinoma bone metastases, hepatic fibrosis, as well as melamine and cyanuric acid induced nephrotoxicity.

Assay Principle

Cell Biolabs' Hydroxyproline Assay Kit provides a convenient colorimetric method for the detection of total hydroxyproline from tissue, plasma, serum, or urine acid-hydrolysates. First, the unknown samples or hydroxyproline standards are added to a 96 well plate. Then, a Chloramine T mixture is added to convert the hydroxyproline to a pyrrole. Finally, a 4-(Dimethylamino)benzaldehyde (DMAB) mixture (also known as Ehrlich's Reagent) is added to the well which reacts with the pyrrole to produce a chromophore (Figure 1) and the absorbance of the plate is read at 540-560 nm. The content of hydroxyproline in the unknown samples is determined by comparison with a predetermined hydroxyproline standard curve. The provided reagents are sufficient for the evaluation of 96 assays including standards and unknown samples.

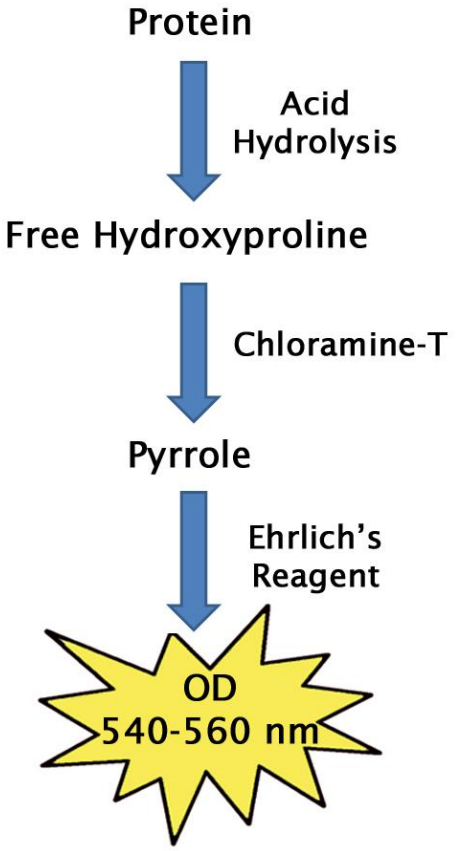

Figure 1. Assay principle.

Related Products

1. STA-670: Homocysteine ELISA Kit
2. STA-671: S-Adenosylhomocysteine (SAH) ELISA Kit
3. STA-672: S-Adenosylmethionine (SAM) ELISA Kit
4. STA-674: Glutamate Assay Kit

Kit Components

1. Hydroxyproline Standard (Part No. 267501): One 100 µL vial containing 1 mg/mL Hydroxyproline.
2. Assay Buffer (Part No. 267502): One 12 mL bottle.
3. Chloramine T Reagent (Part No. 267503): One 600 µL vial.
4. 2X Ehrlich's Concentrate (Part No. 267504): One 5 mL bottle.
5. Ehrlich's Diluent (Part No. 267506): One 5 mL bottle.

Materials Not Supplied

1. 12 N HCl
2. Activated charcoal
3. Water bath or incubator capable of heating to 60°C
4. Oven or autoclave for acid hydrolysis of samples at 95°C
5. 96 well ELISA strips or 96 well microtiter plate
6. 0.6 mL or 1.5 mL microcentrifuge tubes
7. 0.5 mL or 2 mL screw-cap microcentrifuge tubes or Teflon capped, pressure tight vials
8. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
9. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
10. Multichannel micropipette reservoir
11. Microplate reader capable of reading at 540-560 nm

Storage

Upon receipt, store the entire kit at 4ºC.

Preparation of Reagents

- Chloramine T Mixture: Incubate Chloramine T Reagent for 10-15 minutes at 37ºC. Vortex if needed to dissolve completely. For each well to be measured, add 6 µL of Chloramine T Reagent to 94 µL of Assay Buffer. Mix well. Use this mixture within 3 hours of preparation and discard unused Chloramine T Mixture. Aliquot remainder of unused Chloramine T Reagent before returning to 4ºC storage to avoid multiple heat/cold cycles.
- Ehrlich's Reagent: Warm the 2X Ehrlich's Concentrate to room temperature to liquefy. For each well to be measured, mix 50 µL of 2X Ehrlich's Concentrate with 50 µL of Ehrlich's Diluent. Mix well. Use within 3 hours of preparation and discard unused Ehrlich's Reagent.

Preparation of Standard Curve

Prepare a dilution series of Hydroxyproline standards in the concentration range of 0 to 100 µg/mL by diluting the Hydroxyproline Standard in distilled water (Table 1).

Standard Tubes	1 mg/mL Hydroxyproline Standard (µL)	Distilled Water (µL)	Hydroxyproline (µg/mL)
1	10	90	100
2	50 of Tube #1	50	50
3	50 of Tube #2	50	25
4	50 of Tube #3	50	12.5
5	50 of Tube #4	50	6.25
6		50	0

Table 1. Preparation of Hydroxyproline Standards.

Preparation of Samples

All samples must undergo acid hydrolysis before use in the assay. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

- Cells: Resuspend 3-6 x 10^6 cells in distilled water and proceed to Acid Hydrolysis Protocol below.
- Tissue: Homogenize in 100 µL of distilled water for every 10 mg of tissue. Proceed to Acid Hydrolysis Protocol below.
- Urine, plasma, or serum: Use directly in the Acid Hydrolysis Protocol below.

Acid Hydrolysis Protocol for all Samples

1. Transfer 100 µL of sample to one of the following:
    a. A 0.5 mL or 2 mL Teflon capped, pressure tight vial; then add 100 µL of 12 N hydrochloric acid and incubate for 3 hours at 120°C, OR
    b. A 0.5 mL or 2 mL screw-cap microcentrifuge tube; then add 100 µL of 12 N hydrochloric acid and incubate for 24 hours at 95ºC.
2. Let cool briefly, then clarify samples by one of the following methods:
    a. Pass the hydrolzyed sample through a 0.45 µm PVDF syringe filter, OR
    b. Add 5 mg of activated charcoal, mix well by vortexing, centrifuge at 10,000 xg for 5 minutes, recover the supernatant and transfer to a new tube.
3. Proceed directly to the assay or store hydrolyzed samples at 4ºC.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each sample, unknown and standard should be assayed in duplicate.
2. Add 10 µL of unknown acid hydrolyzed samples to separate microcentrifuge tubes.

    Note: If needed, unknown samples may be diluted in water.

3. Evaporate unknown acid-hydrolyzed samples under vacuum to dryness at 60-80ºC for 30-45 minutes. If a vacuum source is not available, evaporation may be performed on a heat block or in an oven or waterbath.

   Note: Unknown samples must be dried to remove any residual HCl that could inhibit the colorimetric assay reaction.

4. Add 10 µL of each hydroxyproline standard to separate tubes.
5. Add 100 µL of the Chloramine T Mixture to each tube.
6. Incubate for 30 minutes at room temperature.
7. Add 100 µL of Ehrlich's Reagent to each tube.
8. Incubate **45** minutes at 60ºC.

   Note: Precipitation may occur during this step or the next step.

9. Transfer all tubes to 4°C and incubate for 5 minutes.
10. Centrifuge all tubes at 6000xg for 15 minutes at room temperature.
11. Transfer 150 µL of the supernatant to separate microplate wells.
12. Read absorbance of each well on a microplate reader using 540-560 nm as the primary wave length.

Example of Results

The following figures demonstrate typical Hydroxyproline Assay Kit results. One should use the data below for reference only. This data should not be used to interpret actual results.

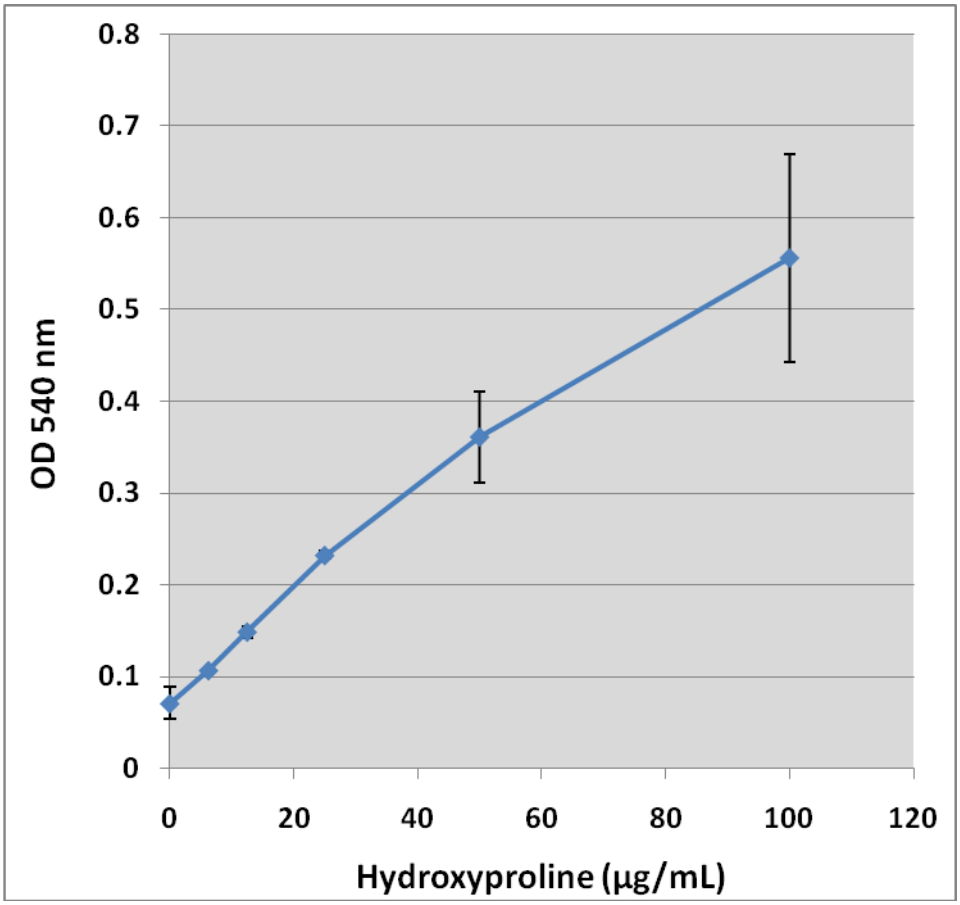

Figure 2: Hydroxyproline Standard Curve.

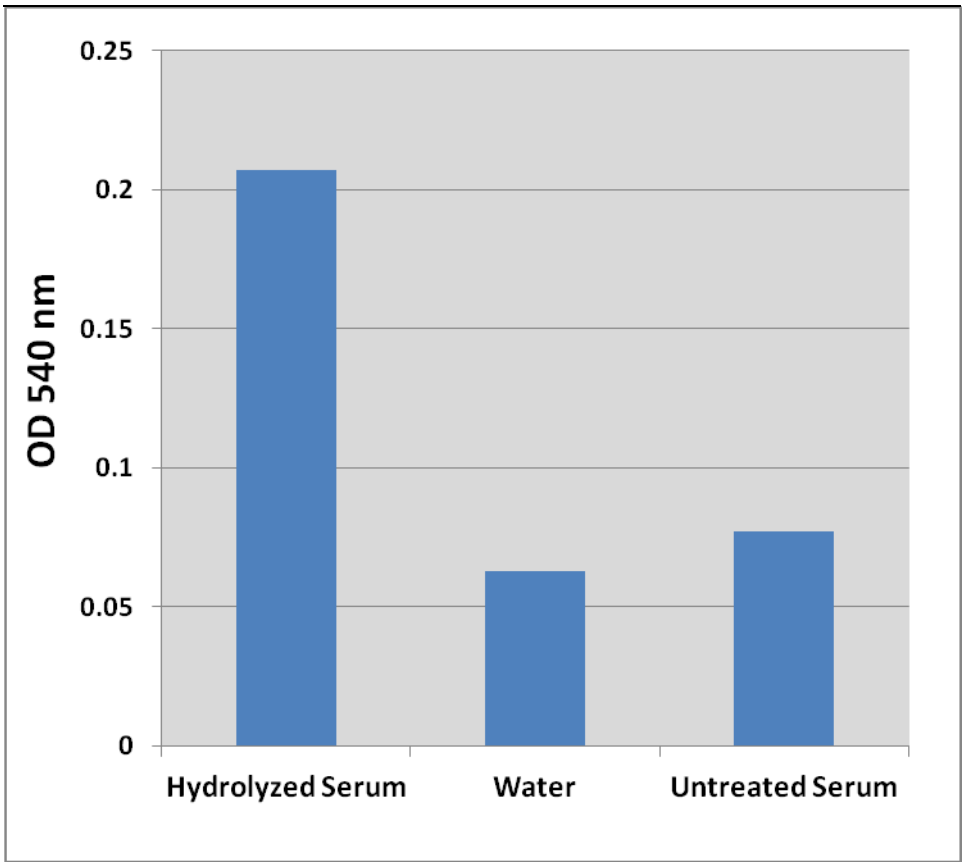

Figure 3: Detection of Hydroxyproline in Human Serum. Pooled human serum (or water as a negative control) was treated by acid hydrolysis according to the Preparation of Samples Section. Samples were tested according to the Assay Protocol.

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Recent Product Citations

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