

Revised Protocol

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Product Manual

# Hydroxyproline Assay Kit (Perchlorate-Free)

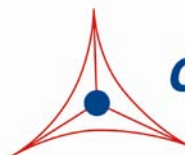
Catalog Number

STA-675

96 assays

**FOR RESEARCH USE ONLY**  
Not for use in diagnostic procedures

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*Creating Solutions for Life Science Research*

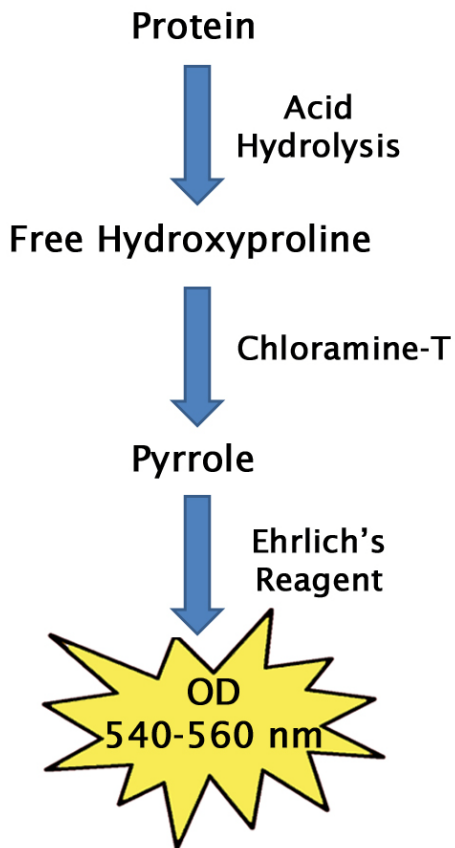
## **Introduction**

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-Adosylmethionine (SAM) ELISA Kit
4. STA-674: Glutamate Assay Kit

### **Kit Components**

1. Hydroxyproline Standard (Part No. 267501): One 100  $\mu$ L 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  $\mu$ L 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 120°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 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 4°C.

## **Preparation of Reagents**

- Chloramine T Mixture: Incubate Chloramine T Reagent for 10-15 minutes at 37°C. 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 4°C 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**

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

- Cells: Resuspend  $3-6 \times 10^6$  cells in distilled water. Transfer 100 μL of cell suspension to a 0.5 mL or 2 mL Teflon capped, pressure tight vial and add 100 μL of 12 N hydrochloric acid. Hydrolyze the sample for 3 hours at 120°C. Let cool briefly and then add 5 mg of activated charcoal\*. Mix well by vortexing and then centrifuge at 10000 xg for 5 minutes. Recover the supernatant and transfer to a new tube. Store unused final sample at 4°C.
- Tissue: Homogenize in 100 μL of distilled water for every 10 mg of tissue. Transfer 100 μL of tissue homogenate to a 0.5 mL or 2 mL Teflon capped, pressure tight vial and add 100 μL of 12 N hydrochloric acid. Hydrolyze the sample for 3 hours at 120°C. Let cool briefly and then add 5 mg of activated charcoal\*. Mix well by vortexing and then centrifuge at 10000 xg for 5 minutes. Recover the supernatant and transfer to a new tube. Store unused final sample at 4°C.
- Urine, plasma, or serum: Transfer 100 μL of sample to a 0.5 mL or 2 mL Teflon capped, pressure tight vial and add 100 μL of 12 N hydrochloric acid. Hydrolyze the sample for 3 hours at 120°C. Let cool briefly and then add 5 mg of activated charcoal\*. Mix well by vortexing and then centrifuge at 10000 xg for 5 minutes. Recover the supernatant and transfer to a new tube. Store unused final sample at 4°C.

*\*Note: If activated charcoal is not available, then omit this step and pass the hydrolyzed sample through a 0.45 μm PVDF syringe filter unit.*

### **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. If an oven is not available for incubating tubes at 60°C, incubate in a waterbath. Transfer 150 µL of the final colorimetric reaction (see step 11 below) to a microplate for final absorbance reading.*

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

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

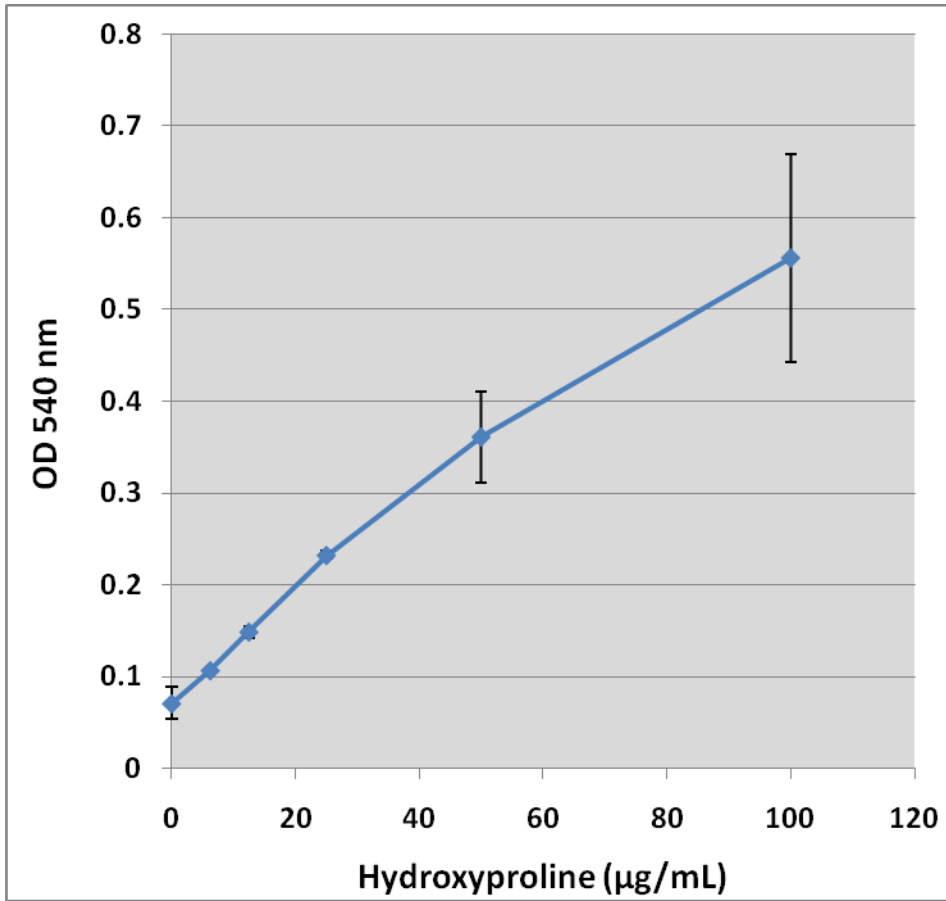
4. Add 10 µL of each hydroxyproline standard to separate tubes.
5. Add 100 µL of the Chloramine T Mixture to each tube.
6. Incubate for 30 minutes at room temperature.
7. Add 100 µL of Ehrlich's Reagent to each tube.
8. Incubate **45** minutes at 60°C.

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

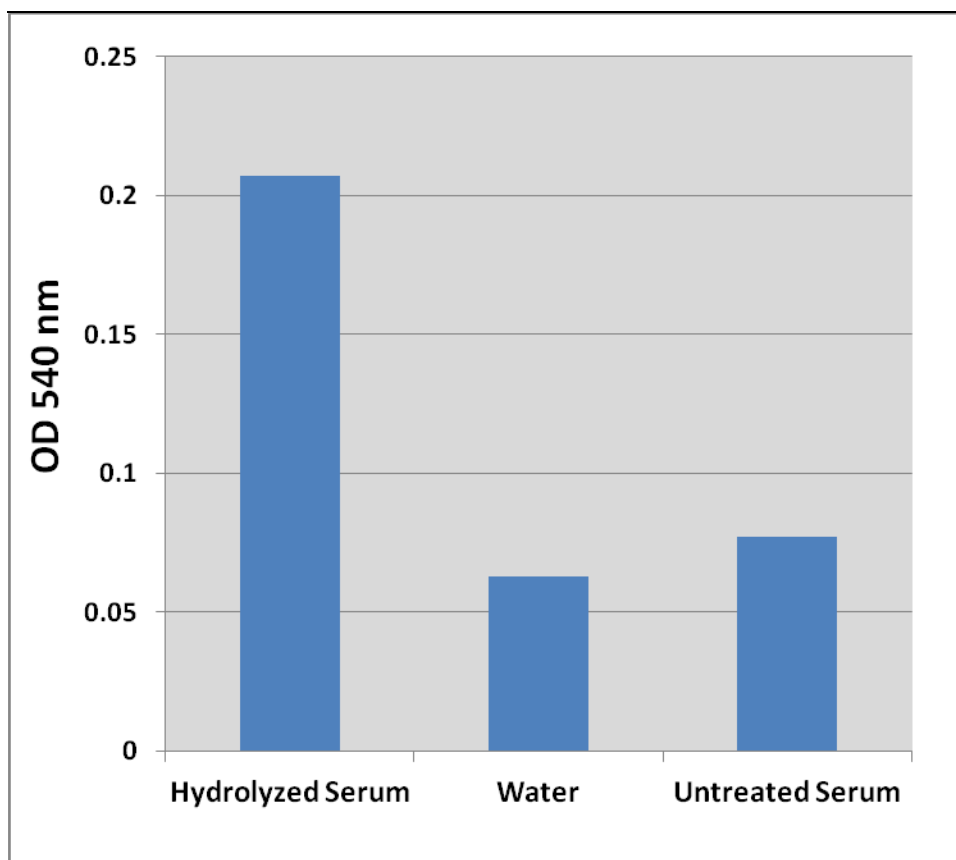
9. Transfer all tubes to 4°C and incubate for 5 minutes.
10. Centrifuge all tubes at 6000xg for 15 minutes at room temperature.
11. Transfer 150 µL of the supernatant to separate microplate wells.
12. Read absorbance of each well on a microplate reader using 540-560 nm as the primary wavelength.

### **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.

## References

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## **Recent Product Citations**

1. Yoshida, T., et al. (2017). Pigment Epithelium-Derived Factor (PEDF) Prevents Hepatic Fat Storage, Inflammation, and Fibrosis in Dietary Steatohepatitis of Mice. *Dig Dis Sci.* **62**(6):1527-1536. doi: 10.1007/s10620-017-4550-x.
2. Liu, H. et al. (2016). Cholesterol 7 $\alpha$ -hydroxylase protects the liver from inflammation and fibrosis by maintaining cholesterol homeostasis. *J Lipid Res.* doi:10.1194/jlr.M069807.
3. Park, H. J. et al. (2016). Roflumilast ameliorates airway hyper-responsiveness caused by diet-induced obesity in a murine model. *Am J Respir Cell Mol Biol.* doi:10.1165/rcmb.2015-0345OC.

## **Warranty**

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