Hydroxyproline Assay Kit

Hydroxyproline Assay Kit measures Hydroxyproline in serum, plasma, urine and cell / tissue lysates.

Catalog number: ARG81311

For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Hydroxyproline is produced by hydroxylation of the amino acid proline by the enzyme prolyl hydroxylase following protein synthesis (as a post-translational modification). The enzyme catalyzed reaction takes place in the lumen of the endoplasmic reticulum. Although it is not directly incorporated into proteins, hydroxyproline comprises roughly 4% of all amino acids found in animal tissue, an amount greater than seven other amino acids that are translationally incorporated.

Hydroxyproline is a major component of the protein collagen, comprising roughly 13.5% of mammalian collagen. Hydroxyproline and proline play key roles for collagen stability. They permit the sharp twisting of the collagen helix. In the canonical collagen Xaa-Yaa-Gly triad (where Xaa and Yaa are any amino acid), a proline occupying the Yaa position is hydroxylated to give a Xaa-Hyp-Gly sequence. This modification of the proline residue increases the stability of the collagen triple helix. It was initially proposed that the stabilization was due to water molecules forming a hydrogen bonding network linking the prolyl hydroxyl groups and the main-chain carbonyl groups. It was subsequently shown that the increase in stability is primarily through stereoelectronic effects and that hydration of the hydroxyproline residues provides little or no additional stability.

In addition to collagen, the mammalian proteins elastin and argonaute 2 have collagen-like domains in which hydroxyproline is formed. Some snail poisons, conotoxins, contain hydroxyproline, but lack collagen-like sequences.

Hydroxylation of proline has been shown to be involved in targeting Hypoxia-inducible factor (HIF) alpha subunit (HIF-1 alpha) for degradation by proteolysis. Under normoxia (normal oxygen conditions) EGLN1 protein hydroxylates the proline at the 564 position of HIF-1 alpha, which allows ubiquitylation by the von Hippel-Lindau tumor suppressor (pVHL) and subsequent targeting for proteasome degradation.

Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen and/or gelatin amount.

Hydroxyproline rich glycoproteins (HRGPs) are also found in plant cell walls. These hydroxyprolines serve as the attachment points for glycan chains which are added as post-translational modifications.

Proline hydroxylation requires ascorbic acid (vitamin C). The most obvious, first effects (gingival and hair problems) of absence of ascorbic acid in humans come from the resulting defect in hydroxylation of proline residues of collagen, with reduced stability of the collagen molecule, causing scurvy.

Increased serum and urine levels of hydroxyproline have also been demonstrated in Paget's disease. [Provide from Wikipedia]

PRINCIPLE OF THE ASSAY

This Hydroxyproline Assay Kit employs a convenient colorimetric method for the detection of total hydroxyproline from serum, plasma, urine and cell / tissue lysates. Samples are hydrolysed by acid before assay. The hydrolysed 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, the 1X Ehrlich's Reagent (4-(Dimethylamino)-benzaldehyde (DMAB)) is added to the well. It reacts with the pyrrole to produce a chromophore and the intensity of the color is measured at a wavelength of 540-560 nm. The concentration of hydroxyproline in the unknown samples is then determined by comparing the O.D of samples to the standard curve. The provided reagents are sufficient for the evaluation of 96 assays including standards and unknown samples.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Assay Buffer	12 ml (Ready-to-use)	4°C
Hydroxyproline Standard (1 mg/ml)	100 µl	4°C
Chloramine T Reagent	0.6 ml	4°C
2X Ehrlich's Concentrate	5 ml	4°C
Ehrlich's Diluent	5 ml (Ready-to-use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 540 - 560 nm
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Deionized or distilled water
- 96 well ELISA strips or 96 well microtiter plate
- 12 N HCl
- 0.5 mL or 2 mL Teflon or polypropylene screw-capped, pressure tight vials.
- Activated charcoal or 0.45 µm PVDF syringe filter unit
- Oven for heating at 60-120°C
- Vacuum to dryness samples
- (Optional) heat block, hot plate

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Ensure complete reconstitution and dilution of reagents prior to use.
- All materials should be equilibrated to room temperature (RT, 22-25°C) few minutes before use.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- It is highly recommended assaying the control and samples in duplicates.

- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

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

Note: If activated charcoal is not available, then omit adding activated charcoal step (step 3 at below) and pass the hydrolyzed sample through a 0.45 µm PVDF syringe filter unit after cooling sample briefly.

Cells:

1. Resuspend 3 - 6 x 10^6 cells in 100 µl distilled water. Transfer 100 µl of cell suspension to a 0.5 mL or 2 mL Teflon capped, pressure tight vial.
2. Add 100 µL of 12 N hydrochloric acid. Hydrolyze the sample for 3 hours at 120°C.
3. Let samples cool briefly on ice and then add 5 mg of activated charcoal. Mix well by vortexing and then centrifuge at 10000 x g for 5 minutes.
4. Collect the supernatant and transfer to a new tube.
5. Store unused final sample at 4°C.

Tissue:

1. Homogenize tissue in 1 ml of distilled water for every 100 mg of tissue. Transfer 100 µL of tissue homogenate to a 0.5 mL or 2 mL Teflon capped, pressure tight vial.
2. Add 100 µL of 12 N hydrochloric acid. Hydrolyze the sample for 3 hours at 120°C.

   Note: Some extremely tough samples such as bone containing tissue or exoskeletal tissue may require heating for longer time for complete

hydrolysis.

3. Let samples cool briefly on ice and then add 5 mg of activated charcoal. Mix well by vortexing and then centrifuge at 10000 x g for 5 minutes.
4. Collect the supernatant and transfer to a new tube.
5. Store unused final sample at 4°C.

Urine, plasma, or serum:

1. Transfer 100 µL of sample to a 0.5 mL or 2 mL Teflon capped, pressure tight vial.
2. Add 100 µL of 12 N hydrochloric acid. Hydrolyze the sample for 3 hours at 120°C.
3. Let samples cool briefly on ice and then add 5 mg of activated charcoal. Mix well by vortexing and then centrifuge at 10000 x g for 5 minutes.
4. Collect the supernatant and transfer to a new tube.
5. Store unused final sample at 4°C.

REAGENT PREPARATION

- **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. . Prepare a master mixture solution to ensure consistency. Use this mixture within 2 hours of preparation and discard unused Chloramine T Mixture. Aliquot remainder of unused Chloramine T Reagent before returning to 4°C storage to avoid repeated freeze-thaw 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.
- **Sample**: If the initial assay found samples contain Hydroxyproline (Hyp) higher than the highest standard or the samples are expected to contain high concentration of Hydroxyproline, the samples can be diluted with diluted in distilled water and then re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account.
- **Standards:** The concentration of Hydroxyproline standard stock of 1 mg/ml. The distilled water serves as zero standard (0 µg/ml), and the rest of the standard serial dilution can be diluted with distilled water as according to the suggested concentration below: 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml.

Dilute Hydroxyproline (Hyp) standard as according to the table below:

Standard	Standard Conc. (µg/mL)	µl of distilled water	µl of standard
S5	100	90	10 of 1 mg/ml Stock
S4	50	50	50 of S5
S3	25	50	50 of S4
S2	12.5	50	50 of S3
S1	6.25	50	50 of S2
S0	0	50	0

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT) before use. Prepare and mix all reagents thoroughly before use. Samples, standards should be assayed in duplicates.

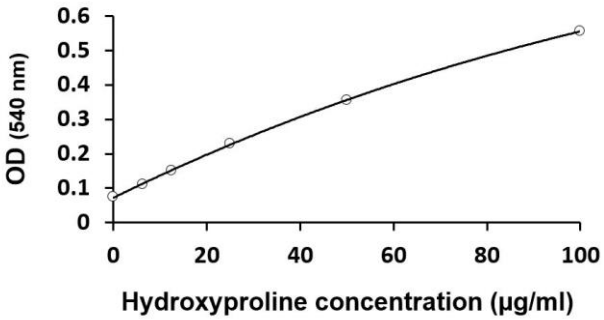
1. Add 10 µL of unknown acid hydrolyzed samples and each hydroxyproline standard to separate microcentrifuge tubes.
   Note: If needed, unknown samples may be diluted in distilled water.
2. Evaporate unknown acid-hydrolyzed samples and standards under vacuum to dryness at 60-80°C for 30-45 minutes. If a vacuum source is not available, evaporation may be performed on a heat block, hot plate or in an oven.
   Note: Unknown samples must be dried to remove any residual HCl that could inhibit the colorimetric assay reaction.
3. Add 100 µL of the Chloramine T Mixture to each tube.
4. Incubate for 30 minutes at room temperature.
5. Add 100 µL of Ehrlich's Reagent to each tube.
6. Incubate 45 minutes at 60°C.
   Note: Precipitation may occur during this step or the next step. If an oven is not available for incubating tubes at 60°C, incubate in a water bath or hot plate.
7. Cool all tubes at 4°C and incubate for 5 minutes.
8. Centrifuge all tubes at 6000 x g for 15 minutes at room temperature.
9. Transfer 150 µL of the supernatant to separate microplate wells.
10. Read absorbance of each well on a microplate reader using 540-560 nm (read at 560 nm is best) as the primary wave length immediately.

CALCULATION OF RESULTS

1. Subtract the mean absorbance value of the blank (S0, Standard #0) from all standard and sample readings. This is the corrected absorbance. Calculate the average absorbance values for each set of standards and samples.
2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean corrected absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
5. According to sample preparation section the samples are acid-hydrolysis by equal part of HCl, so the samples are diluted 2X before assay. The concentration read from the standard curve must be further converted by 2. Please refer the detail for sample preparation procedure as described above.
6. If the samples have been further diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.
7. Hydroxyproline MW: 131.13 g/mol, Conversion: Hydroxyproline (µg/ml) x 7.6 = Hydroxyproline µM.

EXAMPLE OF RESULTS

1. The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



2. Hydroxyproline detection Assay: Hydrolyzed serum, untreated serum and water control were tested with this kit reading the OD at 540 nm.