Technical Bulletin

Hydroxyproline Assay Kit

Catalogue number MAK569

Product Description

Collagen is the major structural protein of the extracellular matrix in many tissues. Hydroxyproline, major component of collagen, makes up about 13.5% of its amino acid composition. Due to its highly restricted distribution in collagen, the hydroxyproline content accurately reflects the amount of collagen. Therefore, quantitating hydroxyproline has been utilized for evaluating tissue fibrosis or collagen deposition.

In the Hydroxyproline Assay Kit, hydroxyproline concentration is determined by the reaction of oxidized hydroxyproline with 4-(dimethylamino) benzaldehyde (DMAB), which results in a colorimetric product (absorption max = 560 nm), proportional to the hydroxyproline present.

Components

The kit is sufficient for 100 colorimetric assays in 96-well plates.

- Oxidation Buffer 10 mL
  Catalogue Number MAK569A
- Chloramine T Concentrate 0.6 mL
  Catalogue Number MAK569B
- Perchloric Acid/Isopropanol Solution 5 mL
  Catalogue Number MAK569C
- DMAB Concentrate in DMSO 4 x 1.67 mL
  Catalogue Number MAK569D
- Hydroxyproline Standard 0.1 mL
  Catalogue Number MAK569E

Reagents and Equipment Required but Not Provided

- 96-well plates, clear, flat bottom. It is recommended to use clear plates for colorimetric assays. (Catalogue number M2936 or equivalent)
  - Cell culture or tissue culture treated plates are not recommended.
- Plate reader that is capable to read wavelength of 560 nm.
- Pipettors and Pipettes
- Vortex Mixer
- Dry bath

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the safety data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped at room temperature. Store at 2–8° C, protected from light. The reagent concentrates are stable as supplied.

Preparation Instructions

Briefly centrifuge vials before opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Oxidation Buffer: Allow buffer to come to room temperature before use.

DMAB Concentrate: Warm to room temperature prior to use. Store protected from light and moisture at 2-8 °C.

Procedure

All samples and standards should be run in technical duplicates or triplicates.

Preparation of Hydroxyproline Standards

1. Dilute 10 µL of the 1 mg/mL Hydroxyproline Standard Solution with 90 µL of water to prepare a 0.1 mg/mL Standard solution.
2. Add 0, 2, 4, 6, 8, and 10 µL of the 0.1 mg/mL Hydroxyproline Standard solution into a 96-well plate, generating 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0 µg/well standards.

Sample Preparation

For serum or urine samples, transfer 100 µL. of sample to a pressure-tight polypropylene vial with cap. Add 100 µL of concentrated hydrochloric acid (HCl, ~12 M), cap tightly, and hydrolyze at 120 °C for 3 hours. Add 4 mg of activated charcoal, mix, and centrifuge at 10,000 × g for 3 minutes. Transfer 10–50 µL of supernatant to a 96-well plate.

Homogenize 10 mg tissue or cells in 100 µL of water and transfer to a pressure-tight polypropylene vial with cap. Add 100 µL of concentrated hydrochloric acid (HCl, ~12 M), cap tightly, and hydrolyze at 120 °C for 3 hours. Mix and centrifuge at 10,000 × g for 3 minutes. Transfer 10–50 µL of supernatant to a 96-well plate. Hydrolysis of samples may result in discoloration. Evaporate all sample wells to dryness under vacuum. Alternatively, place plates in a 60 °C oven to dry samples.

For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the Standard curve.

Note: Endogenous compounds may interfere with the reaction. To ensure the accurate determination of hydroxyproline in the test Samples, it is recommended to set up a spiked sample control for each sample. Spike the control group with 0.4 µg (4 µL) of the hydroxyproline standard.

Preparation of Assay Reagents

The following two assay reagents are stable for 2–3 hours after preparation, and should be prepared after sample preparation, just prior to the start of the assay. It is advised to only make as much reagent as is necessary for the number of samples and standards to be assayed.

Chloramine T/Oxidation Buffer Mixture: 100 µL is required for each reaction well. For each well, add 6 µL of Chloramine T Concentrate to 94 µL of Oxidation Buffer and mix well.

Diluted DMAB Reagent: 100 µL is required for each reaction well. For each well, add 50 µL of DMAB Concentrate to 50 µL of Perchloric Acid/Isopropanol Solution and mix well.

Assay Reaction

1. Add 100 µL of the Chloramine T/Oxidation Buffer Mixture to each sample and standard well. Incubate at room temperature for 5 minutes.
2. Add 100 µL of the Diluted DMAB Reagent to each sample and standard well, and incubate for 90 minutes at 60 °C.
3. Measure the absorbance at 560 nm (A_{560}).

Results

Calculations

The background for the assay is the value obtained for the 0 (blank) hydroxyproline standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtr acted from all readings.

Note: A new standard curve must be set up each time the assay is run. Use the values obtained from the appropriate hydroxyproline standards to plot a standard curve. The amount of hydroxyproline present in the samples may be determined from the standard curve.

Concentration of Hydroxyproline:

Sa/Sv = C

Sa = Amount of hydroxyproline (µg) in unknown sample, as calculated from the calibration curve

Sv = Sample volume (µL) added into the wells

C = hydroxyproline concentration in sample

Note: For spiked samples, correct for any sample interference by subtracting the sample reading from the spiked sample reading:

$$Sa = \frac{(\text{A560})\text{sample X 0.4 ug}}{(\text{A560})\text{spiked control} - (A560)sample}$$

For example, if the calculated hydroxyproline amount of the sample from the calibration curve is 6.24 µg, and the amount of sample added to the well is 40 µL, then:

C = 6.24 nmol / 40 µL

Hydroxyproline concentration in sample = 0.16 µg /µL.

Figure 1.
Example of a hydroxyproline calibration curve.

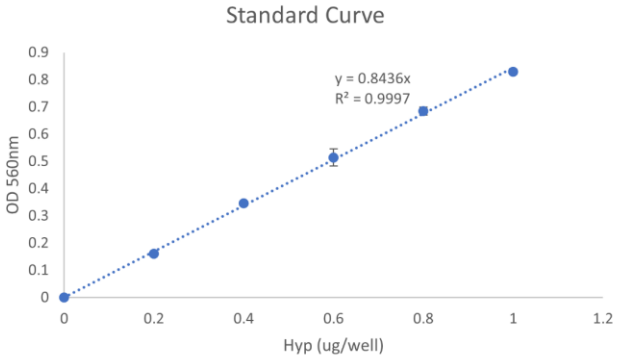
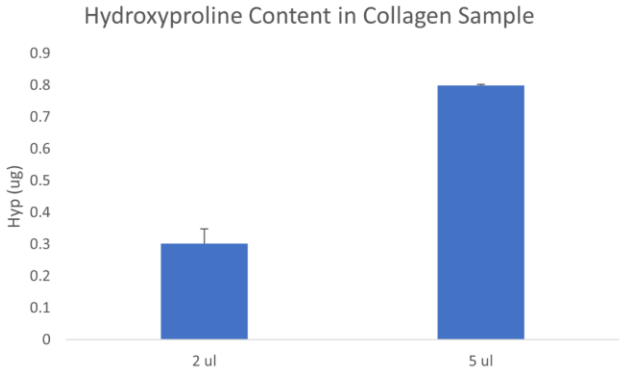


Figure 2.
Collagen sample (catalogue number C4407) was analyzed for hydroxyproline content using the kit. Collagen solution (2.5 mg/mL) was hydrolyzed with HCL 12M. After hydrolysis, the concentration of collagen sample was calculated to be 1.25mg/ml. different volumes of the solution were assayed according to the kit's protocol.



References

1.Woessner J. F., Jr The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys*. **93**: 440–447(1961).

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