Technical Bulletin

Hydroxyproline Assay Kit

Catalogue number MAK008

Product Description

Hydroxyproline (4-hydroxyproline) is a non-proteinogenic amino acid formed by the post translational hydroxylation of proline. Hydroxyproline is a major component of collagen, where it serves to stabilize the helical structure. Because hydroxyproline is largely restricted to collagen, the measurement of hydroxylproline levels can be used as an indicator of collagen content. Conditions that increase collagen turnover can elevate serum and urine hydroxyproline levels.

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 (560 nm) product, proportional to the hydroxyproline present. This kit is suitable for hydroxyproline detection in cell and tissue culture supernatants, urine, plasma, serum, and other biological samples.

Components

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

- Oxidation Buffer 10 mL
  Catalogue Number MAK008A
- Chloramine T Concentrate 0.6 mL
  Catalogue Number MAK008B
- Perchloric Acid/Isopropanol Solution 5 mL
  Catalogue Number MAK008C
- DMAB Concentrate in DMSO 5 mL
  Catalogue Number MAK008D
- Hydroxyproline Standard, 1 mg/mL 0.1 mL
  Catalogue Number MAK008E

Reagents and Equipment Required but Not Provided

- 96-well flat-bottom plate. It is recommended to use clear plates for colorimetric assays. Cell culture or tissue culture treated plates are not recommended.
- Spectrophotometric multiwell plate reader
- Concentrated (37% or - 12 M) Hydrochloric Acid (HCl, Catalog Number 320331 or equivalent)
- Activated charcoal (Catalog Number 242276 or 97876, or equivalent)
- 120 °C Heating block
- Pipette compatible with concentrated HCl
- Centrifugal Evaporator or 60 °C oven
- Pressure-tight polypropylene vial
- (Catalog Number TMO362800-0020, or equivalent)
- Nalgene™ PPCO Low-Profile Caps (Fisher Scientific Catalog Number 03-390-55, or equivalent)

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 on wet ice. Storage at 2–8 °C, protected from light, is recommended. 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 duplicate. Use ultrapure water for the preparation of Samples and Standards and Samples.

Hydroxyproline Standards for Colorimetric Detection

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. 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

To prepare 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 of the hydroxyproline Standard.

Assay

Preparation of Assay Reagents – The following 2 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.

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 (A560).

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 subtracted 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 in unknown sample (µg) from standard curve

Sv = Sample volume (µL) added into the wells

C = Concentration of hydroxyproline in sample

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

$$S_a = \frac{(A_{560})_{\text{sample}}}{(A_{560})_{\text{spiked control}} - (A_{560})_{\text{sample}}} \times 0.4\ \mu g$$

Sample Calculation:

Amount of hydroxyproline (Sa) = 5.84 µg

Sample volume (Sv) = 50 µL

Concentration of hydroxyproline in sample:

5.84 µg/50 µL = 0.1168 µg/µL

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