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Product Information

Hydroxyproline Assay Kit

Catalog Number **MAK008**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

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. Urine and serum hydroxyproline levels can be used as a marker for bone resorption.

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

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.

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.

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.

Procedure
All samples and standards should be run in duplicate. Use ultrapure water for the preparation of 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 PTFE-lined 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 PTFE-lined 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 (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 subtracted from all readings.

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.

Note: A new standard curve must be set up each time the assay is run.

Concentration of Hydroxyproline

$$S_a/S_v = C$$

S_a = Amount of hydroxyproline in unknown sample (μg) from standard curve

S_v = 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 (S_a) = 5.84 μg

Sample volume (S_v) = 50 μL

Concentration of hydroxyproline in sample

$$5.84\ \mu g/50\ \mu L = 0.1168\ \mu g/\mu L$$

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96-well plate used	For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in buffer	Use ultrapure water for sample preparation
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
	Use of improperly stored reagents	Check the storage requirements and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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