Technical Bulletin

Hydroxyproline Assay Kit

Catalogue number MAK463

Product Description

Hydroxyproline is a unique modified amino acid that is found exclusively in several animal proteins, the most prevalent of which is collagen. Collagen is a key structural protein of the extracellular matrix and is common in connective tissues due to its incredible strength. Because of its role as a key structural protein in animals, collagen is crucial for many medical applications, such as tissue transplantation and scaffolding for complex cell cultures. Collagen is also used in the beauty industry, as it can be supplemented to increase tissue rigidity, resulting in skin that appears more youthful.

The Hydroxyproline Assay Kit delivers a safe, simple, and sensitive mean to quantify hydroxyproline in Samples. In the first step of this procedure, hydroxyproline in the sample is oxidized to a pyrrole ring. This compound then reacts with a dye reagent to yield a pink product that can be measured at 560 nm. Hydroxyproline exists almost exclusively in collagen, so hydroxyproline content can be used as a proxy for collagen content.

The linear detection range of the kit is 0.5 µg/mL to 50 µg/mL hydroxyproline in a 96-well plate assay. The kit is suitable for hydroxyproline and collagen detection in biologic (such as serum, cells, urine, and tissue lysate) and cosmetic samples.

Components

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

- Reagent A 1mL
  Catalogue Number MAK463A
- Oxidation Buffer 10mL
  Catalogue Number MAK463B
- Reagent B 10mL
  Catalogue Number MAK463C
- Hydroxyproline Standard 200µL
  (1 mg/mL)
  Catalogue Number MAK463D

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (example., multichannel pipettor)
- 1.5 mL microcentrifuge tubes
- 10 N NaOH and HCl
- Clear flat-bottom 96-well plates. Cell culture or tissue culture treated plates are not recommended.
- Spectrophotometric multiwell plate reader
- Heat block or water bath capable of 100 °C
- Microcentrifuge capable of RCF≥14,000×g

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 components at 2-8 °C.

Preparation Instructions

Briefly centrifuge small vials prior to opening.

Equilibrate all components to room temperature prior to use.

Preparation

Sample Preparation

Collagen Sample Hydrolysis

This kit detects free hydroxyproline. Any Samples containing collagen must be hydrolyzed prior to assay.

1. To hydrolyze Samples, combine 50 µL Sample with 50 µL of 10N NaOH in microcentrifuge tubes.
2. Heat tubes at 100 ºC for 1 hour (NOTE: Solid Samples may require a longer hydrolysis time).
3. Allow tubes to cool to room temperature.
4. Neutralize tubes with 50 µL of 10N HCl.
5. Dilute Samples 1:1 with 150 µL of purified water.
6. Centrifuge tubes for 2 minutes at 14,000×g at room temperature to collect all liquid in the tube and to pellet debris.

Biological Fluid Samples (examples: serum, cells, urine, tissue lysate)

Centrifuge for 5 minutes at 14,000 ×g at room temperature to remove any particulates. A serial dilution in purified water may be needed to ensure the Sample falls within the kit's detection limit.

Standard Curve Preparation

1. Prepare a 50 µg/mL Hydroxyproline Standard by mixing 20 µL of the 1 mg/mL Hydroxyproline Standard with 380 µL of purified water. Prepare Hydroxyproline Standards in 1.5 mL microcentrifuge tubes according to Table 1.

Table 1.

Preparation of Hydroxyproline Standards

No.	50 ug/mL Hydroxyproline Standard	Purified Water	Hydroxyproline (µg/mL)
1	100 µL	-	50
2	60 µL	40 µL	30
3	30 µL	70 µL	15
4	-	100 µL	0 (Blank)

2. Transfer 20 µL standards and samples into separate wells of a clear, flat-bottom 96-well plate.

Reaction

1. For each well, prepare 98 µL of Reaction Mix according to Table 2

Table 2.

Preparation of Reaction Mix

Reagent	Reaction Mix
Reagent A	8 µL
Oxidation Buffer	90 µL

2. Add 90 µL of Reaction Mix to each well.
3. Tap plate to mix and incubate for 10 minutes at room temperature.
4. Add 90 µL of Reagent B to all wells. When Reagent B is added, the wells will become turbid. Pipette up and down until the turbidity dissipates.
5. Incubate the plate for 90 minutes at 37 °C in the plate reader or an incubator.

Measurement

At the 90-minute time point, read the optical density (OD) of each well at 560 nm.

Results

1. Subtract the blank (Standard #4) OD value from the remaining Standard OD values (ΔOD).
2. Plot the ΔOD values against Standard concentrations.
3. Determine the slope of the Standard curve and calculate the hydroxyproline concentration of the Sample:

Hydroxyproline (µg/mL) =

$$\frac{OD_S - OD_B}{\text{Slope } (\mu g/mL^{-1})} \times DF$$

where:

OD_S = OD reading of Sample at 560 nm

OD_B = OD reading of Blank (Standard#4) at 560 nm

DF = Sample Dilution factor (DF=1 for undiluted Samples)

Note: The dilution factor for the Samples treated using the Collagen Hydrolysis protocol is 6 (50 µL of Sample + 50 µL of 10N NaOH + 50 µL of 10N HCl + 150 µL of purified water).

Conversions: 50 µg/mL equals 5 mg/dL, or 50 ppm. Hydroxyproline constitutes 13% of total collagen weight on average. 1µg/mL Hydroxyproline is equivalent to 1/0.13 or 7.69 µg/mL collagen.

Figure 1.

Typical Hydroxyproline Standard Curve

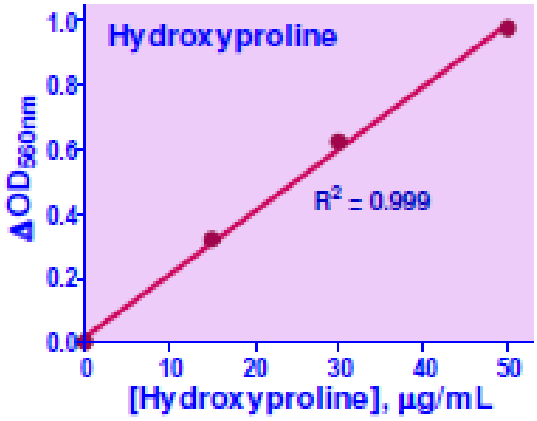
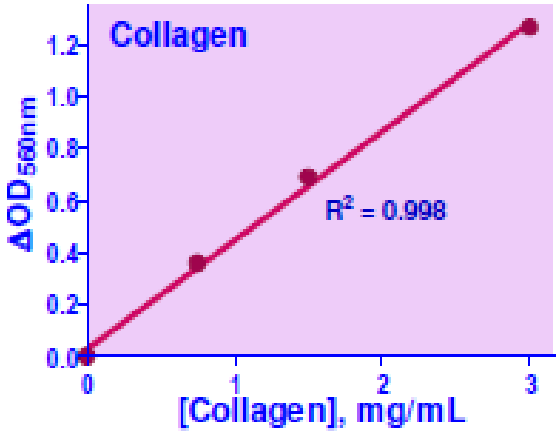


Figure 2:

Typical Collagen assay in 96-well plate. Collagen solution (Catalogue Number C4243) was digested per the protocol and the collagen content was determined to be 3.3 ± 0.2 mg/mL.



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