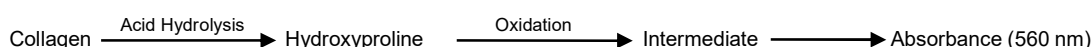


Total Collagen Assay Kit (Colorimetric)

(Catalog #BN00495; 100 assays; Store at -20°C)

I. Introduction:

Collagen is the most abundant insoluble protein found in the extracellular matrix and connective tissues. It can be found in skin, tendons, bone, cartilage, muscle, vitreous humor and ligaments, among other tissues. There are more than sixteen - well characterized types of collagens, but types I, II and III collagen comprise more than 80% content in mammals. The triple-helical structure of collagen is quite unique: it consists of a repeating pattern of a basic trimer: Glycine-Proline-Hydroxyproline. In cells, collagens are secreted as procollagens and these chains are transported into the Endoplasmic Reticulum, where, numerous post-translational modifications lead to the formation of a triple helix with disulfide bonds. Excessive production of collagen is linked to pathological conditions including liver cirrhosis, lung fibrosis, and tumor growth. Assay Genie's Collagen Assay Kit is a simple and sensitive assay to detect small amounts of collagens in a variety of samples. The assay is based on the acid hydrolysis of samples to form hydrolysates and Hydroxyproline. This released Hydroxyproline gets oxidized to form a reaction intermediate, which further in the reaction, forms a chromophore (Abs 560 nm). The assay is simple, sensitive and specific for collagen and can detect as low as 0.5 µg of collagen in a variety of samples such as tissue homogenates, biological fluids and purified proteins.



II. Application:

- Measurement of collagen in various sample types.

III. Sample Types:

- Mammalian tissues
- Protein/peptide hydrolysates
- Serum
- Urine

IV. Kit Contents:

Components	BN00495-100	Cap Code	Part Number
Oxidation Buffer	10 ml	WM	BN00495-1
Chloramine T Concentrate	0.6 ml	Red	BN00495-2
Perchloric Acid/Isopropanol Solution	5 ml	NM	BN00495-3
DMAB Concentrate (in DMSO)	5 ml	Amber	BN00495-4
Collagen I Standard (2 mg/ml)	0.1 ml	Yellow	BN00495-5

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer
- Hydrochloric Acid (concentrated)
- Acetic Acid (Glacial)
- For hydrolysis: Polypropylene Vials

VI. Storage Conditions and Reagent Preparation:

Store the kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **Chloramine T Reagent:** For each well to be analyzed, add 6 µl of Chloramine T Concentrate to 94 µl of Oxidation Buffer and mix well.
- **DMAB:** For each well to be analyzed, add 50 µl of DMAB Concentrate to 50 µl of Perchloric acid/Isopropanol Solution and mix well. Keep on ice, protected from light.

Note: The reagent concentrates are stable as supplied. Once the concentrates have been diluted to working concentration, they are only good for 2-3 hours, so prepare reagents as necessary for the number of samples and standards to be quantified.

VII. Collagen Assay Protocol:

1. Sample Preparation: Tissue or protein/peptide, tissue samples (i.e. lung) should be homogenized in ddH₂O, using 100 µl ddH₂O for every 10 mg of tissue. To a 100 µl of sample homogenate, add 100 µl concentrated HCl (~12 M, not provided) in a pressure-tight Teflon capped vial. Hydrolyze samples at 120 °C for 3 hrs (**See note c**). Urine: hydrolyze samples with equal volumes of concentrated HCl (~12 N; i.e. 100 µl Urine + 100 µl HCl) in a pressure-tight, teflon capped vial. After homogenization, clarify samples with activated charcoal by adding 4 mg of activated charcoal. Vortex and centrifuge at 10000 x g for 3 min. to remove precipitate & activated charcoal. Repeat if needed. Transfer 10-30 µl of each hydrolyzed sample to a 96-well plate and evaporate to dryness under vacuum/on a hot plate/in an oven.

Notes:

- For unknown samples, we suggest performing a pilot experiment & testing different sample dilutions to ensure the readings are within the Standard Curve Range.
- Endogenous compounds may interfere with the reaction. To ensure accurate determination of collagen in the test samples, we recommend spiking samples with a known amount of collagen I Standard (4.0 µg)

- c. For sample hydrolysis, polypropylene vials yield best results. We recommend Assay Genie's Polypropylene Vials and Caps Cat. No.
- 2. Standard Preparation:** Prepare 1.0 mg/ml Collagen I Standard by adding 50 μ l of 2 mg/ml Type I Standard to 50 μ l of 0.02 M Acetic Acid. Add 0, 2, 4, 6, 8, and 10 μ l of 1.0 mg/ml Collagen I Standard into a series of pressure-tight Teflon capped vials to generate 0, 2, 4, 6, 8 and 10 μ g of collagen/well. Adjust the volume to 10 μ l/vial with 0.02 M Acetic Acid. Add 10 μ l of 12 M HCl to the pressure-tight Teflon capped vial and hydrolyze at 120°C for 3 hrs. Place vials on ice and spin down contents. Transfer the contents of each vial (~15 μ l) to a 96-well plate and evaporate to dryness under vacuum/on a hot plate/in an oven.

Notes:

- Use diluted Collagen standard (1.0 mg/ml) within 24 hours. Prepare fresh diluted stocks prior any experiments.
 - Do not expose the microplate to extreme temperatures. If a hot plate/oven is used, we recommend placing the microplate at 70 °C until the contents are completely evaporated
- 3. Reaction:** Add 100 μ l of the Chloramine T reagent to each sample and standard and incubate at room temperature for 5 min. Add 100 μ l of the DMAB reagent to each well and incubate for 90 min. at 60 °C.
- 4. Measurement:** Measure absorbance at 560 nm in a microplate.
- 5. Calculation:** Correct background by subtracting the value derived from the 0 Collagen standard from all readings (the background reading can be significant and must be subtracted). Plot the Standard curve. Apply the sample readings to the standard curve to get the collagen amount in the reaction wells (B).

$$\text{Total Collagen Concentration (C)} = B/V \times D \text{ } \mu\text{g}/\mu\text{l}$$

Where: **B** is amount of Collagen in the sample well from Standard Curve (μ g)
V is sample volume added into the reaction well (μ l)
D is sample dilution factor

Note: For spiked samples, correct for any sample interference by using the following equation:

$$\text{Collagen amount in spiked sample well (B)} = \frac{\text{OD}_{\text{sample (corrected)}}}{(\text{OD}_{\text{sample+Collage Std (corrected)}}) - (\text{OD}_{\text{sample (corrected)}})} * \text{Collagen spike } (\mu\text{g})$$

Hydroxyproline MW: 131.13 g/mol

Hydroxyproline Content in Type I Collagen (from rat tail): 12-14% by weight

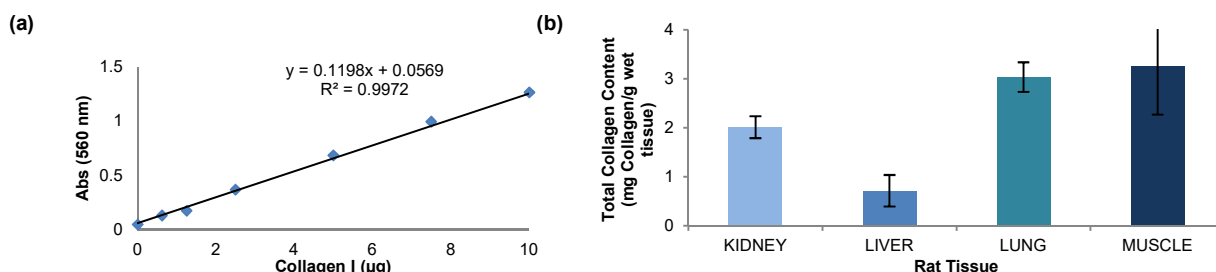


Figure: (a) Collagen Standard Curve (0-10 μ g). (b) Estimation of Total Collagen Concentration in rat tissues. Rat kidney, liver, lung and muscle samples were homogenized with ddH₂O and hydrolyzed with 12 M HCl for 3 hrs at 120 °C. Precipitates were removed by centrifugation (10000 x g, 3 min.). Thirty microliters of the hydrolyzed samples were assayed and kit was performed according to the protocols. Collagen Content (mg Collagen/g wet tissue): Kidney: 2.01 \pm 0.22; Liver: 0.71 \pm 0.32; Lung: 3.03 \pm 0.3; Muscle: 3.25 \pm 0.98.

FOR RESEARCH USE ONLY! Not to be used on humans.