Soluble Collagen Assay Kit (Fluorometric) (BN00764)

(Catalog # BN00764; 100 Reactions; Store at -20°C)

I. Introduction:

Collagen is the most abundant protein in mammals, accounting for nearly 30% of body protein content. Collagen is an extracellular scaffolding protein found in skin, tendons, bone, cartilage, ligaments and muscles, amongst other tissues. There are more than twenty different characterized types of collagen, but the fibrilous types I, II and III comprise more than 90% of the total collagen in mammals. Collagen has a triple helical structure, consisting of three 'preprocollagen' strands with a repeating glycine- and proline-rich tripeptide sequence (Gly-Pro-X) and small end-cap domains called registration peptides. Nascent preprocollagen strands undergo enzymatic post-translational hydroxylation at proline and lysine residues prior to secretion to the extracellular milieu via the golgi apparatus. Once outside the cell, the dangling registration peptides are cleaved off, forming tropocollagen, the monomeric precursor to mature collagen fibers. While mature collagen exists as an insoluble web of extensively crosslinked fibrils, tropocollagen is soluble in dilute acidic solutions. Many diseases are believed to affect collagen synthesis, fibrilization and turnover, including tumor invasion and metastasis, rheumatoid arthritis, cardiopulmonary fibrosis and muscular dystrophy. Assay Genie's Soluble Collagen Assay Kit allows for quantification of newly synthesized (acid-soluble) collagen levels in tissues and cultured cell medium. The assay involves extraction of soluble collagen in acetic acid, followed by enzymatic degradation of collagen into glycine-rich oligopeptides, which are quantified using a fluorogenic reagent and developer solution that selectively react with the *N*-terminal glycine fragments to form a stable fluorescent complex (Ex/Em = 376/468 nm). The assay is more sensitive and selective than dye-binding (Picrosirius Red) soluble collagen assays, is simple to perform and has a linear range from 0.05 – 2 µg collagen per well (2.5 µg/ml to 100 µg/ml in a 20 µl sample volume).

II. Applications:

- Estimation of newly synthesized collagen in various biological samples and cultured cells

III. Sample Type:

- Mammalian soft tissues (lung, liver, *etc.*) homogenized in dilute acetic acid
- Cultured cells (extracellular matrix of adherent cell lines) and cell culture medium (secreted extracellular collagen)

IV. Kit Contents:

Components	BN00764	Cap Code	Part Number
Collagen Assay Buffer	25 ml	WM	BN00764-1
Collagenase Enzyme Mix	1 vial	Green	BN00764-2
Detection Reaction Buffer	10 ml	NM	BN00764-3
Peptide Labelling Reagent	1 vial	Red	BN00764-4
Developer Solution	500 µl	Orange	BN00764-5
Collagen I Standard (2 mg/ml)	100 µl	Yellow	BN00764-6

V. User Supplied Reagents and Equipment:

- Multiwell microplate spectrofluorometer
- White 96-well plates with flat bottom
- 0.5 M acetic acid and 0.5 M sodium hydroxide (NaOH)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the Collagen Assay Buffer and Detection Reaction Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **Collagenase Enzyme Mix:** Reconstitute with 220 µl Collagen Assay Buffer, divide into aliquots and store at -20°C. Avoid multiple freeze/thaw cycles. Use within 2 months after reconstitution.
- **Peptide Labelling Solution:** Reconstitute with 220 µl ddH_2O. Divide into aliquots and store at -20°C, **protected from light**. Prior to use, warm solution to room temperature and vortex thoroughly. Use within 2 months after reconstitution.
- **Developer Solution:** Provided as a ready-to-use solution. Divide into aliquots and store at -20°C, **protected from light**. Prior to use, warm solution to room temperature and vortex thoroughly. Use within 2 months after opening.
- **Collagen I Standard (2 mg/ml):** Provided as a 2 mg/ml stock solution of solubilized Type I collagen from rat tail tendon in dilute acetic acid. Store at +4°C and allow solution to come to room temperature prior to use.

VII. Soluble Collagen Assay Protocol:

1. Sample Preparation:

Soft Tissues and Adherent Cultured Cells (Acid Soluble Collagen):

a. *For soft tissues:* Rinse tissue samples with ice-cold ddH_2O or PBS to remove any residual blood, blot dry and mince with clean scissors. Transfer minced tissue to a pre-chilled glass bead (Dounce) homogenizer and add 1 ml of ice-cold 0.5 M acetic acid per ~100 mg of wet tissue. Homogenize tissue **on ice**, transfer tissue homogenate to a microfuge tube, vortex thoroughly and incubate at 4°C overnight with gentle stirring/rotation (***maximal solubilization of collagen is achieved after ~8 hours***).

For adherent cells: Remove culture medium, detach cells manually using a rubber cell scraper and collect harvested cells in PBS. Pellet cells by centrifugation and aspirate PBS. Resuspend cell pellet in 1 ml of ice-cold 0.5 M acetic acid per ~1 x 10^7 cells. Transfer cell slurry to a pre-chilled Dounce homogenizer and homogenize **on ice**. Transfer homogenate to a microfuge tube, vortex thoroughly and incubate at 4°C overnight gentle stirring/rotation (***maximal solubilization of collagen is achieved after ~8 hours***).

b. Following overnight incubation, centrifuge the homogenate at 10000 x *g* for 15 min at 4°C and transfer the acidic supernatant to a new microfuge tube. Neutralize acidic sample extract by adding an equal volume of 0.5 M NaOH to the supernatant (*i.e.* mix 500 µl of acidic supernatant and 500 µl of 0.5 M NaOH).

c. Add 2-20 µl of neutralized sample extract to desired well(s) in a white 96-well plate. For each test sample, prepare *two parallel sample wells*, with one well serving as a sample background control. Adjust the volume of all wells to 80 µl/well with Collagen Assay Buffer.

Notes:

- It is important to keep samples chilled during the homogenization procedure. Heat generated by homogenization can cause denaturation and crosslinking of soluble tropocollagen fibrils, rendering them insoluble in acid solutions.
- Soluble collagen levels and the effectiveness of acid solubilization can vary tremendously between tissues. Collagen present in "tough" tissues (such as cartilage or connective tissue) is highly crosslinked and tends to be resistant to acid solubilization.

Cell Culture Medium (Secreted Soluble Collagen):

d. Collagen secreted into cell culture medium may be assayed directly, without the need for acid solubilization. Remove a sample of culture medium without detaching cells, centrifuge at 10000 x *g* for 15 min at 4°C to pellet any debris and transfer the clarified supernatant to a new microfuge tube. Add 10-20 µl of clarified medium to two parallel wells (one well will serve as a sample background control) and adjust the volume to 80 µl/well with Collagen Assay Buffer.

2. **Standard Curve Preparation:** Prepare a 0.2 mg/ml collagen solution by adding 20 µl of the 2 mg/ml Collagen I Standard to 180 µl of ddH_2O. Add 0, 2, 4, 6, 8, and 10 µl of the 0.2 mg/ml working solution into a series of wells, generating 0, 0.4, 0.8, 1.2, 1.6 and 2 µg of collagen/well. Adjust the volume of all standard wells (including the 0 µg/well reagent blank) to 80 µl/well with Collagen Assay Buffer.

3. **Reaction Preparation:**

a. Prepare a working solution of collagenase by diluting the reconstituted Collagenase Enzyme Mix stock solution at 1:10 ratio with Collagen Assay Buffer. Prepare enough of the working solution to add 20 µl to each reaction well (for 10 reactions, mix 20 µl of Collagenase Enzyme Mix stock and 180 µl of Collagen Assay Buffer). Add 20 µl of collagenase working solution to test sample and standard wells. For sample background control wells, add 20 µl of Collagen Assay Buffer only. Incubate plate at 37°C for 60 min.

b. Following incubation, prepare detection reaction solution by diluting the reconstituted Peptide Labelling Reagent stock in Detection Reaction Buffer at a 1:30 ratio. Prepare enough of the working solution to add 75 µl to each reaction well (for 10 reactions, mix 25 µl of Peptide Labelling Reagent stock and 725 µl of Detection Reaction Buffer). Add 75 µl of detection reaction solution to all test sample and standard wells (including sample background control wells) and incubate plate (protected from light) at 37°C for 5 min.

c. Prepare developer working solution by diluting the Developer Solution stock in ddH_2O at a 1:10 ratio. Prepare enough diluted developer solution to add 25 µl to each reaction well (for 10 reactions, mix 25 µl of Developer Solution stock and 225 µl of ddH_2O). Add 25 µl of detection reaction solution to all test sample and standard wells (including sample background control wells). Incubate the plate (protected from light) at 37°C for 15 min with gentle orbital shaking to ensure well contents are effectively mixed.

4. **Measurement:** Measure the fluorescence (Ex/Em = 376/468 nm) of all test sample and standard curve wells in endpoint mode.

5. **Calculations:** For the collagen standard curve, subtract the reagent blank (0 µg/well collagen standard) fluorescence (RFU) value from all standard readings, plot the background-subtracted values and calculate the slope of the standard curve. For test samples, subtract the corresponding sample background control well RFU value from the sample reading ($F = RFU_{Sample} - RFU_{BC}$) and apply the background-subtracted fluorescence (F) to the standard curve to get B µg of soluble collagen in the well.

$$\textbf{Sample Soluble Collagen Concentration} = \frac{\boldsymbol{B}}{\boldsymbol{V}} \times \boldsymbol{D} = \textbf{µg/µl}$$

Where: ***B*** is the amount of collagen, calculated from the standard curve (in µg)
V is the volume of sample added to the well (in µl)
D is the sample dilution factor (if applicable, D=1 for undiluted samples)

Note: The calculation above gives the amount of collagen in the sample added to the well. The dilution factor D is only needed if the sample is diluted *after* the neutralization step. When calculating the amount of collagen in the original sample homogenate, remember to account for the 2-fold dilution that occurs during neutralization of the acidic homogenate.

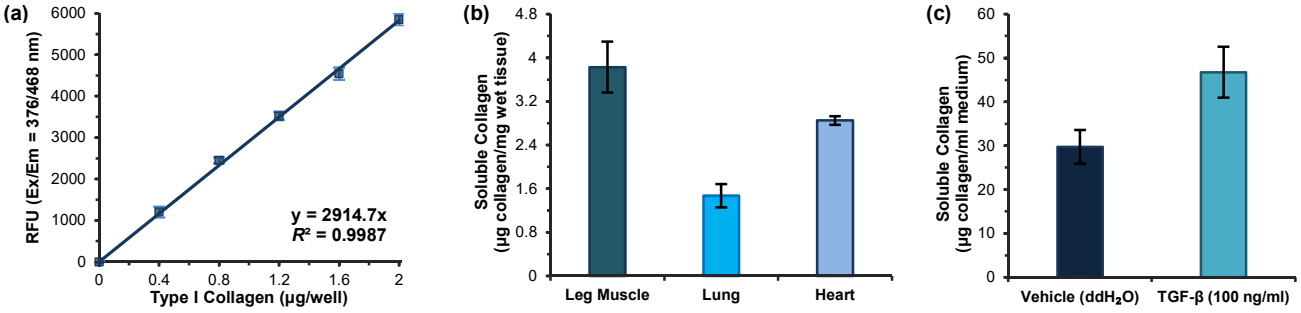

Figure: (a) Collagen I Standard curve. **(b)** Estimation of acid-soluble collagen content in rat tissues. Rat leg muscle, lung and heart samples were homogenized in 0.5 M acetic acid, incubated overnight at 4°C to solubilize collagen and neutralized with 0.5 M NaOH. Collagen levels (calculated as µg collagen/mg wet tissue) for the samples were: 3.83 ± 0.47 µg/mg for muscle, 1.47 ± 0.22 µg/mg for lung and 2.85 ± 0.08 µg/mg for heart. **(c)** Estimation of secreted soluble collagen in cultured cell growth medium (DMEM/F12 medium). 3T3-L1 fibroblasts were grown to ~80% confluence in T-75 flasks and then treated with either vehicle or TGF-β, a cytokine known to stimulate synthesis and secretion of tropocollagen fibrils. After 48 hours of treatment, the culture medium was removed, centrifuged to remove debris and was assayed directly (each 10 µl clarified medium, undiluted). TGF-β treatment resulted in a roughly 1.5-fold increase in collagen secretion. Data are mean ± SEM of 3 replicates, assayed according to the kit protocol.

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