Collagenase (Collagen Degradation/Zymography) Assay Kit (Fluorometric) (BN00725)

(Catalog # BN00725; 100 assays, Store kit at -20°C)

I. Introduction:

Collagenases, members of the matrix metalloproteinase (MMP) family, are endopeptidases that digest native collagen in the triple helix region and are involved in the physiological and pathological turnover of connective tissues. Collagens are the major fibrous component of animal extracellular connective tissue. Bacterial collagenases differ from vertebrate collagenases in that they exhibit broader substrate specificity. Collagenases may play a role in facilitating tumor cell invasion of the extracellular matrix at multiple stages of the metastatic process. Collagenases have also been approved for medical uses for the treatment of Dupuytren's contracture, Peyronie's disease and wound healing. Collagenase activity is usually detected by small peptide-based activity assays which may suffer from lack of substrate specificity. Other methods for collagenase activity include collagen Zymography where samples are electrophoresed on a collagen-containing SDS-PAGE, and further renatured in a suitable buffer for 12-16 h. The zymogram is subsequently stained, and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme. Such methods are laborious, time-consuming and may lead to the loss of enzymatic activity as renaturation may not be completely reversible. Assay Genie's Collagenase Activity Assay Kit utilizes a hybrid approach for the detection of collagenase activity by employing a highly quenched collagen substrate which upon cleavage by a suitable collagenase releases a fluorophore, which can be easily quantified using a conventional microplate reader. This method is substrate-specific, simple, fast, high-throughput adaptable and amenable to the sensitive detection of collagenase activity (as low as 0.6 mCDU for bacterial collagenase) in biological samples.

II. Applications:

- Detection of collagenase activity in biological samples such as tissue, cell lysates, etc.

III. Sample Type:

- Recombinant protein, tissue, cell lysates, etc.

IV. Kit Contents:

Components	BN00725	Cap Code	Part Number
Collagenase Assay Buffer	25 ml	WM	BN00725-1
Cell Lysis Buffer	25 ml	NM	BN00725-2
Enzyme Positive Control	10 µl	Green	BN00725-3
Collagenase Substrate	1 Vial	Orange	BN00725-4
FITC Standard (5 mM)	10 µl	Yellow	BN00725-5

V. User Supplied Reagents and Equipment:

- 96-well Clear/Black/White well plate (The black plate will yield lowest background while white plate will yield highest background fluorescence).
- Multi-well spectrofluorometer

VI. Storage Conditions and Reagent Preparation:

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

- **Collagenase Assay Buffer**: Bring to room temperature before use. Store at -20°C.
- **Collagenase Substrate**: Reconstitute in 220 µl of DI water. Mix well by pipetting up and down. Vortex if necessary. Unused substrate can be stored at -20°C by covering it with aluminum foil or transferring it to an amber vial.
- **Enzyme Positive Control**: Aliquot and store at -20°C. Thaw on ice before use. Avoid repeated freeze/thaw.

VII. Collagenase Assay Protocol:

1. **Sample Preparation:** Homogenize fresh or frozen tissue (~5-10 mg) or cells (1-2 x 10^6) with 100 µl Cell Lysis Buffer and incubate on ice for 5 min. Centrifuge the homogenate at 16,000 X g, 4°C for 10 min. Transfer the clarified supernatant to a fresh pre-chilled tube and keep on ice. *Measure the amount of protein in the lysate or purified enzyme using BCA Protein Assay Kit - Reducing Agent Compatible*. Add 1-50 µl of lysate or purified enzyme into desired well(s) in a white96-well plate. If necessary, dilute the lysate with Collagenase Assay buffer. For Positive Control, dilute 2 µl of Enzyme Positive Control with 18 µl of Collagenase Assay Buffer and use 1-10 µl/well. Adjust the volume of Samples and Positive Control to 50 µl/well with Collagenase Assay Buffer.

   Notes:

   a. The kit is designed to work with *active Collagenase* enzymes only. If the sample contains inactive zymogen forms of collagenase, it can be activated with p-aminophenylmercuric acetate (APMA) or other activators. The conditions for activation of each enzyme should be determined empirically by following appropriate testing protocol (Shapiro *et. al., J. Bio. Chem.* **1995**, 270 (11), 6351-6356).

   b. We recommend using the tissue/cell homogenate immediately to measure the Collagenase activity. If desired, snap freeze the lysate and store at -80°C.

   c. For unknown samples, we suggest doing pilot experiment and testing 3-5 different amounts of samples to ensure the readings are within the Standard Curve range.

d. To induce higher collagenase expression, cells can be grown in the presence of Phorbol myristate acetate (10-50 ng/ml), lysed and tested directly in the assay (Hersh *et. al*., *Biochem*., **1986**, 25 (17), 4750-4757).

e. Optional: For samples having background, prepare parallel sample well(s) as sample background control. Use same amount of tissue/cell homogenate or purified enzyme as in the sample well. Adjust the final volume to 100 µl with Collagenase Assay Buffer.

2. **Standard Curve Preparation:** Prepare 50 µM of FITC Standard by diluting 2 µl of 5 mM FITC Standard with 198 µl of Collagenase Assay Buffer. Mix well by pipetting up and down, vortex vigorously for 30 s. Add 0, 2, 4, 6, 8, and 10 µl of diluted 50 µM FITC Standard into a series of wells in a 96-well white plate and adjust the final volume to 100 µl/well with Collagenase Assay Buffer to generate 0, 100, 200, 300, 400, and 500 pmol/well of FITC Standard respectively. Mix well and measure the fluorescence at Ex/Em= 490/520 nm in an end point mode at 37 °C.

3. **Collagenase Substrate Mix**: Prepare 50 µl of Collagenase Substrate Mix per well as given below:

48 µl Collagenase Assay Buffer
2 µl Reconstituted Collagenase Substrate

Dissolve the Substrate Mix by vigorous vortexing. Add 50 µl of Substrate Mix solution into each Sample, and Positive Control well.

Note: Do not add Substrate Mix to the sample Background Control and Standard wells.

4. **Measurement**: Mix well and measure the fluorescence at Ex/Em 490/520 nm in kinetic mode at 37 °C for 1-2 h. Choose two time points (t_1 & t_2) where the corresponding RFUs (RFU_1 and RFU_2) are in a linear range. Calculate ΔRFU and Δt and obtain ΔRFU/Δt as RFU/min for each Sample including background control. Subtract the value of RFU/min of background from each Sample to obtain net RFU/min (**B**).

5. **Calculations:**

a. FITC Standard Curve: Obtain change in the RFU (ΔRFU) by subtracting fluorescence of the 0 Standard Controls from those containing all standards. Plot the ΔRFU against pmol of FITC Standard. The plot should be linear; determine the slope **A** (ΔRFU/pmol) of the curve.

b. Samples: Using RFU/min of each Sample, calculate Sample Collagenase activity using following equation.

$$\textbf{Sample Collagenase Activity}\left(X, \frac{\mathbf{U}}{\mathbf{ml}}\right) = \frac{B \times 1000}{A \times C} \times \textit{Dilution Factor}$$

$$\textbf{Sample Collagenase Activity}\left(\frac{\mathbf{U}}{\mathbf{mg}}\right) = \frac{X}{P}$$

where, **B** = Sample Collagenase Activity as calculated (RFU/min),
A = Slope of the FITC standard curve (ΔRFU/pmol),
C = µl of Sample used in the assay,
P = Protein concentration in the lysate (mg/ml).
1000 = Conversion Factor (1000 µl ≡ 1 ml)

Unit Definition: 1 U is the amount of Collagenase required to cleave the Collagenase Substrate and release 1 pmol of Fluorescein per min under the conditions of the assay.

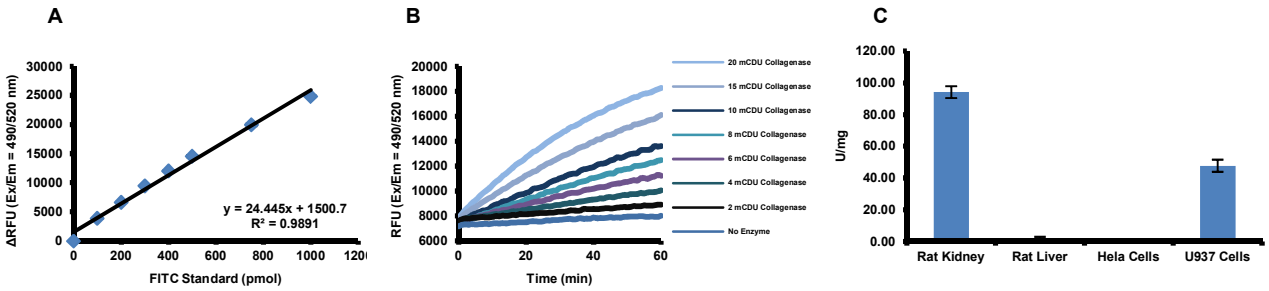

Figure: FITC Standard Curve (A), Collagenase activity with different amounts of Enzyme Positive Control (B), and in rat kidney, liver lysates along with Hela and U937 cell lysates (C) are shown in the figure (n = 3). The assays were performed according to the kit protocol.

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