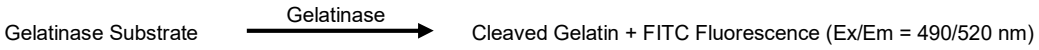
Gelatinase (Gelatin Degradation/Zymography) Assay Kit (Fluorometric) (#BN00686)

(Catalog #BN00686; 100 assays, Store kit at -20°C)

I. Introduction:

Gelatinases are a type of matrix zinc-dependent metalloproteases (MMPs) that degrade gelatins and a variety of other extracellular matrix proteins. These enzymes are synthesized as latent zymogens that are activated by proteolysis and inhibited by tissue inhibitors of metalloproteases (TIMPs). Two mammalian gelatinases, Gelatinase A (MMP-2) and Gelatinase B (MMP-9), are critical for basement membrane degradation and are highly upregulated in variety of tumor cells. Gelatinase activity is usually detected by small peptide-based activity assays which may suffer from lack of substrate specificity. Other methods for gelatinase activity include gelatin Zymography where samples are electrophoresed on a gelatin-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 Gelatinase Activity Assay Kit utilizes a hybrid approach for the detection of gelatinase activity by employing a highly quenched gelatin substrate which upon cleavage by a suitable gelatinase 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 gelatinase activity (as low as 0.06 mCDU for bacterial collagenase) in biological samples.



II. Applications:

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

III. Sample Type:

- Recombinant protein, tissue, cell lysates, etc.

IV. Kit Contents:

Components	BN00686	Cap Code	Part Number
Gelatinase Assay Buffer	25 ml	WM	BN00686-1
Cell Lysis Buffer	25 ml	NM	BN00686-2
Enzyme Positive Control	10 µl	Green	BN00686-3
Gelatinase Substrate	1 Vial	Red	BN00686-4
FITC Standard (5 mM)	10 µl	Yellow	BN00686-5

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.

- **Gelatinase Assay Buffer**: Bring to room temperature before use. Store at -20°C.
- **Gelatinase 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. Gelatinase 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 white 96-well plate. If necessary, dilute the lysate with Gelatinase Assay buffer. For Positive Control, dilute 2 µl of Enzyme Positive Control with 18 µl of Gelatinase Assay Buffer and use 1-10 µl/well. Adjust the volume of Samples and Positive Control to 50 µl/well with Gelatinase Assay Buffer.

   Notes:

   a. The kit is designed to work with active Gelatinase enzymes only. If the sample contains inactive zymogen forms of gelatinase, 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 Gelatinase 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 gelatinase expression, cells can also be grown in the presence of Phorbol myristate acetate (10-50 ng/ml), lysed and tested directly in the assay (Shin *et. al.*, *Exp. Mol. Med.*, **2003**, 39 (1), 97-105).

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 Gelatinase Assay Buffer.

2. **Standard Curve Preparation:** Prepare 50 µM of FITC Standard by diluting 2 µl of 5 mM FITC Standard to 200 µl of Gelatinase 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 Gelatinase 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. **Gelatinase Substrate Mix**: Prepare 50 µl of Gelatinase Substrate Mix per well as given below:

    48 µl Gelatinase Assay Buffer
    2 µl Reconstituted Gelatinase 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 $\Delta RFU/\Delta t$ 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 Gelatinase activity using following equation.

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

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

where, **B** = Sample Gelatinase 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 Gelatinase required to cleave the Gelatinase Substrate and release 1 pmol of Fluorescein per min under the conditions of the assay.

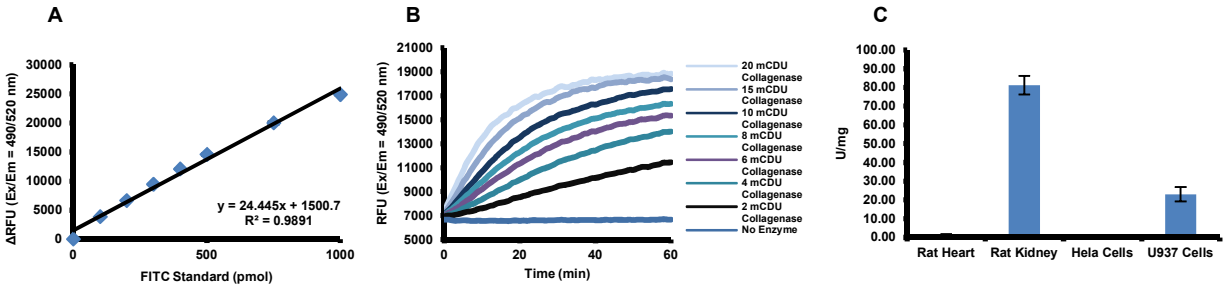


Figure: FITC Standard Curve (A), Gelatinase activity with different amounts of Enzyme Positive Control (B), and in rat heart, kidney lysates along with Hela and U937 cell lysates (C) are shown in the figure (n = 3). The assays were performed according to the kit protocol.

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