Deubiquitinase Activity Assay Kit (Fluorometric) (BN00721)

(Catalog # BN00721; 100 assays; Store kit at -20˚C)

I. Introduction:

Cell activity and viability is tightly regulated by controlling the production and degradation of the thousands of different proteins in the cell. The proteasome is responsible for the majority of cellular protein degradation; however, drugs targeting the proteasome can have side effects caused by the lack of specificity associated with inhibiting the proteasome itself. Altering the ubiquitination state of target proteins is thus appealing as an alternative approach. Modification of the ubiquitin-mediated proteasome pathway has been shown to be a valid mechanism for treating a variety of diseases, all of which involve dysregulation of cellular proteostasis. As such, it is imperative that these ubiquitination signals also be reversible. The enzymes responsible for cleavage, and hence removal of ubiquitin from ubiquitinated proteins, are known as de-ubiquitinating enzymes, or DUBs. They are proteases that hydrolyze the isopeptide bond between an ubiquitin moiety and a lysine residue on its target protein. By removing the ubiquitin molecule, the protein escapes the fate of proteasomal degradation and remains a viable factor in the cell. Assay Genie's Deubiquitinase Activity Assay Kit provides a straight-forward and general measure of deubiquitinase activity by utilizing a fluorescent deubiquitinase substrate to detect activity as low as 0.25 µU with purified enzyme.

$$\text{Fl-Substrate} \xrightarrow{\text{DUB}} \text{Cleaved Substrate + Fl (Fluorescence (Ex/Em = 350/440 nm)}$$

II. Applications:

- Measurement of DUB activity in various tissues/cell extracts
- Determination of DUB activity associated with pathological conditions
- Characterization of activity of purified DUB enzymes

III. Sample Type:

- Purified recombinant protein
- Cell and tissue lysate

IV. Kit Contents:

Components	BN00721	Cap Code	Part Number
DUB Assay Buffer	25 ml	WM	BN00721-1
1 M DTT	100 µl	Green	BN00721-2
DUB Substrate (in DMSO)	25 µl	Red	BN00721-3
DUB Positive Control	1 vial	Orange	BN00721-4
AMC Standard (1 mM)	100 µl	Yellow	BN00721-5
White 96-well Half-Area Plate	1 plate	-	BN00721-6

V. User Supplied Reagents and Equipment:

- Multi-well spectrophotometer (ELISA reader)

VI. Storage Conditions and Reagent Preparation:

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

- **DUB Assay Buffer**: Ready to use. Warm to room temperature before use. Store at -20°C.
- **1 M DTT**: Ready to use. Warm to room temperature before use. Store at -20°C.
- **DUB Substrate and AMC Standard**: Ready to use. Warm DUB Substrate and AMC Standard to room temperature before use. Light sensitive. Do not expose components to light for extended periods of time. Store at -20°C. Use within six months.
- **DUB Positive Control**: Reconstitute with 22 µl of DUB Assay Buffer with DTT to prepare the stock solution. Aliquot & store at -80°C. Avoid repeated freeze/thaw. Use within two months.

VII. DUB Activity Assay Protocol:

1. **Sample Preparation:** Add 1 µl of the supplied 1 M DTT per ml DUB Assay Buffer for a 1 mM final DTT concentration. The Assay Buffer is now ready to use. Make as much as necessary for number of experiments being run. For tissue samples, add 50 µl ice-cold DUB Assay Buffer with 1 mM DTT per mg of sample (wet weight) . Homogenize on ice using a Dounce homogenizer. To prepare cell lysate, resuspend cells in ice-cold Assay Buffer with DTT (10^5 cells per 50 µl) and homogenize in a dounce homogenizer. Centrifuge lysate (tissue or cell) at 10,000 X g for 5 min. at 4°C. Collect the supernatant. Add 5- 10 µl into a well of the provided half-area 96-well plate. For the positive control reaction, use 2 µl of the reconstituted Positive Control. Adjust the volume of each reaction to 40 µl with DUB Assay Buffer (with DTT).

   Notes:

   a. For unknown samples, we suggest trying several dilutions to ensure results are in the linear range of the Standard Curve.

   b. For samples having high background, prepare parallel sample background controls (see step 3).

2. **AMC Standard Curve Preparation:** To prepare AMC Standard, dilute 10 µl of 1 mM AMC Standard into 990 µl DUB Assay Buffer with DTT to obtain a 10 µM stock concentration. Add 0, 2, 4, 6, 8, and 10 µl of diluted standard into a series of wells in a 96-well plate and adjust the final volume to 50 µl/well with the DUB Assay Buffer with DTT to generate 0, 20, 40, 60, 80, and 100 pmol/well AMC Standard. Mix well. Measure fluorescence (Ex/Em = 350/440 nm).

3. **Substrate Mix:** Dilute stock solution to working concentration by adding 25 µl substrate to 1075 µl DUB Assay Buffer with DTT for the working stock (dilution factor = 1:44). If lower numbers of wells (for example, 20) are needed, 5 µl of DUB substrate can be diluted into 215 µl DUB Assay Buffer with DTT. To initiate reaction, add 10 µl substrate mix to each well. Measure Fluorescence at 25°C in kinetic mode.

   Notes:

   a. Use diluted substrate within 4 hours.

   b. No Substrate (Background) Control: For lysates, prepare a parallel sample background reaction by omitting the substrate mix and instead adding 10 µl DUB Assay Buffer with DTT.

4. **Measurement:** Immediately after addition of substrate, measure the fluorescence in a kinetic mode at 25°C for at least 30 min. (Ex/Em = 350/440 nm).

   Note:

   a. Incubation time depends on the DUB activity in samples. We recommend measuring the fluorescence in kinetic mode, and choosing two time points (T_1 & T_2) in the linear range to calculate the DUB activity of the samples.

   b. The AMC Standard Curve can be read in Endpoint mode (i.e., at the end of the incubation time).

5. **Calculation:** Subtract 0 Standard reading from all readings. Plot the AMC Standard Curve. If sample background control reading is significant, subtract the background control reading from its paired sample reading. Calculate the DUB activity of the test sample: $\Delta RFU = RFU_2 - RFU_1$. Apply the ΔRFU to the AMC Standard Curve to get B pmol of AMC generated during the reaction time ($\Delta t = t_2 - t_1$).

   Sample DUB Activity = B/(Δt X V) x D = pmol/min/ml = µU/ml

   Where: **B** = AMC amount from Standard Curve (pmol).
   Δt = reaction time (min.)
   V = sample volume added into the reaction well (ml)
   D = Dilution Factor

Unit Definition: 1 Unit is defined as the amount of DUB that can cleave 1 µmol of substrate/min under the assay conditions at 25°C.

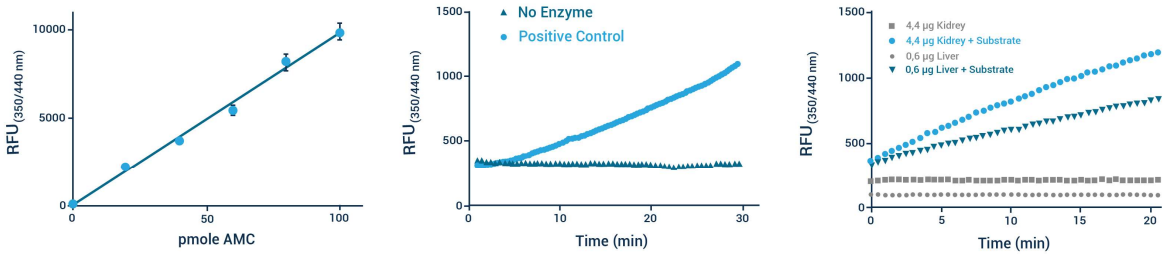

Figure: (a) AMC Standard Curve; (b) Time course using positive control DUB as described; (c) Example of determination of DUB activity in tissue lysates. Rat tissue samples (10 mg each) were resuspended in DUB Assay Buffer with DTT (100 µl), homogenized, and clarified by centrifugation. The DUB activities for Rat Kidney and Liver lysates, in mU/mg, were determined to be 0.76 and 3.31, respectively. Assays were performed following the kit protocol.

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