CREATIVE BIOMART®
Assay Kit

DUB Activity Assay Kit

Product Information

Common Name

DUB

Cat.No. Kit-2527

Product Overview

DUB Activity Assay Kit facilitates the rapid, robust measurement of deubiquitinating enzyme activity in vitro. The kit utilizes a high purity, fluorogenic substrate (ubiquitin-AMC) together with suitable calibration standards and controls for the accurate and sensitive assessment of DUB activity. Continuous kinetic or end-point assays can be performed in a 96-well plate format for multi-sample analysis. Each kit contains sufficient material for one full 96-well plate assay set-up to be run. Ubiquitin-AMC is not a suitable substrate for all DUBs. Compatibility must be determined by the end user.

Kit Components

DUB Assay Buffer (10X), 1 vial/1 ml, -20°C
Control DUB enzyme (USP2 Catalytic Domain), 1 vial/25 µl, -80°C
Ubiquitin-AMC, 1 vial/25 µl, -80°C
AMC Assay Reagent, 1 vial/100 µl, -20°C
DTT (1M) Assay Reagent, 1 vial/1 ml, -20°C
96-Well Cover Sheet 1 cover RT
Half Volume 96-Well Plate (white) 1 plate RT

Materials Required but Not Supplied

1. A plate reader with the ability to measure fluorescence using an excitation wavelength of 355-365 nm and an emission wavelength of 455-465 nm.
2. A source of pure water; glass distilled water or deionized water is acceptable.

Technical Notes

- The final volume of the assay is 50 µl in all the wells.
- All reagents except the DUB Positive Control and samples must be equilibrated to room

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temperature before beginning the assay.
• It is not necessary to use all the wells on the plate at one time.
• We recommend assaying samples in duplicate, but it is the user's discretion to do so.
• The assay is performed at room temperature.
• Monitor the fluorescence with an excitation wavelength of 355-365 nm and an emission wavelength of 455-465 nm.

Preparation

Reagent Preparation
1. DTT (1M) Assay Reagent
This vial contains 1M DTT. Once thawed, the reagent is ready to use and should be stored at -20°C, limiting freeze-thaw cycles.
2. DUB Assay Buffer
This vial contains 1 ml of a 10X Buffer Solution. Once thawed, add 9 ml pure water to make a 1X DUB Assay Buffer. Add 10 µl of 1 M DTT Assay Reagent to the 1X DUB Assay Buffer. The final concentration of DTT in the buffer is 1 mM. This buffer should be used in the assay and for diluting reagents. After addition of DTT, the buffer should be used within the same day or stored at -20°C, limiting freeze-thaw cycles.
3. DUB Positive Control
This vial contains 10 µM of USP2 catalytic domain positive control. Mix 10 µl of the DUB positive control with 990 µl 1X DUB Assay Buffer containing DTT to make a 100 nM control solution. Then mix 100 µl of the 100 nM control solution with 400 µl 1X DUB Assay Buffer containing DTT to make a 20 nM control solution. The original 10 µM DUB Positive Control can be stored long term at -80°C, limiting freeze-thaw cycles.
4. Ubiquitin-AMC
This vial contains Ubiquitin-AMC in DMSO. Mix 21.8 µl of UbiquitinAMC with 478.2 µl 1X DUB Assay Buffer containing DTT. NOTE: The final concentration of substrate in the assay as described below is 500 nM.
5. AMC Assay Reagent
This vial contains 1 mM AMC in DMSO. The reagent is ready to use to prepare the AMC standard curve.

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Standard Preparation

Mix 25 µl of the AMC Assay Reagent with 475 µl of 1X DUB Assay Buffer containing DTT to yield a concentration of 50 µM. Mix 10 µl of this 50 µM standard with 490 µl of 1X DUB Assay Buffer containing DTT to yield a 1 uM AMC stock.

To prepare the standard curve, label eight microcentrifuge tubes. Aliquot 300 µl 1X DUB Assay Buffer containing DTT to tube A and 200 µl 1X DUB Assay Buffer containing DTT to tubes B-H. Transfer 100 µl of the 1 uM AMC stock to tube A and mix throughly. Serially dilute the standard by removing 200 µl from tube A and transferring it to tube B; mix throughly. Next, remove 200 µl from tube B and transfer it to tube C; mix throughly. Repeat this process for tubes D-G. Tube H should only contain 1X DUB Assay Buffer containing DTT and will be used as a blank. Use standards immediately do not store.

Assay Protocol

1. Standard Wells - add 50 µl of standard (tubes A-H) to the designated wells on the plate.
2. Read the fluorescence in a plate reader using an excitation wavelength of 355-365 nm and an emission wavelength of 455-465 nm. This will allow you to establish an appropriate gain for detecting the entire range of the standards. This gain will then be used when assaying samples.
3. DUB Positive Control Wells - add 40 µl of 1X DUB Assay Buffer containing DTT and 5 µl 20 nM DUB Positive Control to at least two wells.
4. Background Wells - add 45 µl of 1X DUB Assay Buffer containing DTT to at least two wells.
5. Sample Wells - add 40 µl of 1X DUB Assay Buffer containing DTT and 5 µl of sample to at least two wells.
6. Cover plate and incubate for 15-30 minutes at room temperature.
7. Remove plate cover and initiate reactions by quickly adding 5 µl of Ubiquitin-AMC to all wells being used.
8. Read the fluorescence in a plate reader every minute for 20-60 minutes using an excitation wavelength of 355-365 nm and an emission wavelength of 455-465 nm.

Analysis

Plot the Standard Curve

1. Determine the average fluorescence of the standards.

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2. Plot the average fluorescence values (from step 1 above) of each standard as a function of the final concentration of AMC.

Determine DUB Activity

1. Determine the average fluorescence of each sample.
2. Determine the change in fluorescence (RFU) per minute for the sample by:

a. Plotting the fluorescence values as a function of time to obtain the slope (rate) of the linear portion of the curve.

OR

b. Select two points on the linear portion of the curve and determine the change in fluorescence during that time using the following equation:

RFU/min = [RFU (Time 2) - RFU (Time 1)]/ [Time 2 (min) - Time 1 (min)]

3. Calculate DUB activity using the equation below. One unit is defined as the amount of enzyme that will cause the formation of 1 pmol of AMC per minute at room temperature.

DUB activity (pmol/min/ml) = (RFU/min) / Standard curve slope (RFU/nM) x Sample dilution

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