DUB Activity Assay Kit

Item No. 15981

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
600184	DUB Assay Buffer (10X)	1 vial/1 ml	-80°C
600185	Control DUB enzyme (USP2 catalytic domain)	1 vial/10 µl	-80°C
600186	Ubiquitin-AMC	1 vial/25 µl	-80°C
600187	AMC Standard	1 vial/1.75 µg	-80°C
600188	96-Well Plate (white; non-binding)	1 plate	Room temperature

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

E-Mail: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section on page 3 and used before the expiration date indicated on the outside of the box. *NOTE: Store kit at -80°C to ensure stability and activity. The 96-well plate can be stored at room temperature. Once dissolved, AMC Standard solutions can be aliquoted and stored at -80°C for up to three months protected from light. For storage of kit component working solutions see* ***Assay Preparation*** *section on page 7. Avoid multiple freeze-thaws.*

Materials Needed But Not Supplied

1. Microplate reader capable of measuring fluorescence using excitation and emission wavelengths of 360 and 460 nm, respectively
2. Microfuge tubes (1.5 ml)
3. A source of 'UltraPure' water. Water used to prepare all reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. Dithiothreitol (DTT) - prepare solutions fresh
5. Dimethyl sulfoxide (DMSO)

INTRODUCTION

Background

Conjugation of ubiquitin to proteins (ubiquitination) plays a fundamental role in the regulation of cellular function through biological events involving, but not limited to cell cycle, differentiation, immune responses, DNA repair, chromatin structure, and apoptosis. The ubiquitin signaling system includes a large family of cysteine proteases known as deubiquitinating enzymes (DUBs) that are responsible for the removal of ubiquitin from modified proteins. This regulatory process allows optimal levels of cellular ubiquitin to be maintained by recycling ubiquitin attached to inappropriate targets, removing and disassembling polyubiquitin chains, and processing proteins prior to their degradation by the proteasome. DUBs in general exhibit a wide range of ubiquitination type (mono- or polyubiquitination) and polyubiquitin chain linkage substrate specificities. In addition, they can be partnered with various interacting proteins to facilitate increased diversity in specificity and DUB activation. DUBs have been implicated in a number of human diseases including various forms of cancer and neurodegeneration. As such, they are attractive targets for potential therapeutic intervention *via* the development of suitable inhibitors and modulators.

About This Assay

Cayman's 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.

This kit can be used to:

1. Screen potential inhibitors and activators for activity against specific DUB enzymes.
2. Assess performance of DUBs of interest with ubiquitin-AMC substrate allowing relevant kinetic data to be generated (*e.g.*, K_M, k_{cat}, k_{cat}/K_M).
3. Evaluate performance of DUB activators and inhibitors enabling relevant kinetic data to be determined (*e.g.*, IC_{50}/EC_{50}, % inhibition/activation, K_i).
4. Optimize assays for specific DUBs to facilitate their use in high throughput screening (HTS).
5. Demonstrate novel, putative DUB enzymes have ubiquitin-AMC processing activity.

NOTE: Please read the protocol in its entirety before starting. Each kit contains sufficient material for one 96-well plate of assays, including controls, and optional calibration standards. Ubiquitin-AMC is not a suitable substrate for all DUBs. Compatibility must be determined by end user.

PRE-ASSAY PREPARATION

Assay Preparation

Working solutions.

1. DUB Assay Buffer Preparation

Dilute the supplied DUB Assay Buffer 10-fold with dH_2O as required to give 1X DUB Assay Buffer for use in assays and preparation of diluted solutions. After dilution, 1X DUB Assay Buffer can be stored for one week at 4°C; freeze for long term storage.

2. Ubiquitin-AMC (Ub-AMC)

Recommend 500 nM Ub-AMC assay concentration as a starting point.

- Ub-AMC substrate supplied at 1 mg/ml in DMSO (114.4 µM).
- Add 21.8 µl stock Ub-AMC to 478.2 µl 1X DUB Assay Buffer to give 500 µl of 5 µM Ub-AMC which is sufficient for ~100 DUB assays at the 500 nM Ub-AMC assay concentration.
- Smaller volumes of 5 µM working solution can be prepared by 22.9X dilution in 1X DUB Assay Buffer.

Storage: Stock and working solutions should be stored at -80°C.

3. Control DUB Enzyme (USP2 CD)

Recommend 10 nM control DUB enzyme assay concentration.

- USP2 CD supplied at 10 µM in 1X DUB Assay Buffer.
- Add 10 µl of 10 µM USP2 CD to 990 µl 1X DUB Assay Buffer to make a 100 nM control DUB enzyme solution.
- Smaller volumes of 100 nM working solution can be prepared by 100X dilution in 1X DUB Assay Buffer.

Storage: 10 µM control DUB enzyme solution can be stored long term at -80°C. The 100 nM control DUB enzyme solution can be stored for up to three months at -80°C.

4. Sample DUB Enzyme

Recommend 10 nM control DUB enzyme assay concentration.

- Optimal assay concentration for specific DUB enzymes will need to be determined by the user.
- Prepare appropriate sample DUB enzyme working solution concentrations by diluting in 1X DUB Assay Buffer; for example, generate a 100 nM working solution for further dilution to a 10 nM final assay concentration.

5. AMC Standard (optional)

- AMC Standard supplied as a solid (1.75 µg).
- Prepare a 160 µM AMC Standard stock solution by dissolving solid in 62.5 µl DMSO.
- Add 10 µl of 160 µM AMC Standard stock solution to 90 µl 1X DUB Assay Buffer to give a 16 µM working solution.

Storage: AMC standard solutions can be stored at -80°C protected from light.

Assay Notes

End Point Assays (linear response)

In order to run meaningful end point assays, for example to screen for DUB activators and inhibitors, the assay conditions used for a specific deubiquitinating enzyme must give a linear response (RFU *vs.* time) over the course of the assay. It is recommended that assays are optimized using the continuous analysis approach prior to application in end point assay set-ups.

DUB Activation by DTT

1X DUB Assay Buffer contains 1 mM DTT. However, some DUBs may require DTT activation prior to use. In this case inclusion of DTT to a final concentration of 10 mM in 1X DUB Assay Buffer is recommended, followed by the standard 15-30 minutes incubation at assay temperature prior to addition of Ub-AMC.

Activators/Inhibitors

Pre-incubate at desired concentration with DUB enzyme in assay mixture at assay temperature for 15-30 minutes prior to initiation of the enzyme reaction by addition of Ub-AMC.

AMC standard curve (optional)

Plotting an AMC standard curve allows DUB activity assay response to be converted from relative fluorescence units (RFU) to defined units (*e.g.*, pmol AMC) if required for subsequent kinetic data analysis.

Control reactions

- Substrate only control: No DUB enzyme - used to adjust readings for background intrinsic substrate fluorescence and any auto-hydrolysis of Ub-AMC.
- Positive control: USP2 CD - demonstrates assay working/components active.
- Inhibitor control: Test enzyme inhibition with general DUB inhibitor such as ubiquitin aldehyde.
- Vehicle control: Solvent for activator/inhibitor compounds (*e.g.*, DMSO) - determines effect, if any, of carrier for test compounds on assay performance.

Assay Optimization

Optimal assay conditions for specific deubiquitinating enzymes must be determined by the user. Adjustment of the following parameters may facilitate this process:

- Sample DUB enzyme concentration: 100 pM-100 nM.
- Ubiquitin-AMC concentration: 0.1-20 µM.
- Assay incubation (end point) or monitoring (continuous) time: 15-60 minutes.
- Pre-incubation with 10 mM DTT for 15-30 minutes.
- Assay temperature - increase from room temperature to 30-37°C.
- Activator/inhibitor concentration and pre-incubation time.

ASSAY PROTOCOL

Pipetting Hints

- Use different tips to pipette each reagent.
- Avoid introducing bubbles to the well.
- Do not expose the pipette tip to the reagent(s) already in the well.

Performing the Assay

It is recommended that all samples, controls and standards be run in triplicate. Ensure Ub-AMC solution is equilibrated to assay temperature before addition to the wells of the plate.

1. Set-up the microplate reader for recording at 360 nm excitation and 460 nm emission.
2. Prepare the required number of sample/control DUB assays (50 µl final volume) in an appropriate number of wells by addition of assay components in the order listed in the table below.
3. Use the plate configuration table provided to aid in proper sample, control, and standard identification.
4. Mix components gently and incubate the plate at room temperature for 15-30 minutes.
5. Add Ub-AMC to initiate assays and mix reagents by shaking the plate gently for 15 seconds.

Component	Sample DUB Enzyme	Control DUB Enzyme	Substrate only (blank)
1X DUB Assay Buffer*	40	40	45
100 nM sample DUB enzyme	5	-	-
100 nM USP2 CD	-	5	-
Incubation	Incubate for 15-30 minutes at room temperature		
5 µM Ub-AMC	5	5	5

Table 1. Buffer preparation summary
*Adjust DUB assay buffer volume to allow for DUB enzyme, Ub-AMC and activator/inhibitor volume used.

Measure the Fluorescence Signal:

1. **For continuous kinetic reading:**

   Commence time-course assay data acquisition immediately upon addition of Ub-AMC substrate. Monitor RFU response for 15-60 minutes taking regular readings (see Figure 1 for USP2 CD example assay).

2. **For end point reading:**

   Incubate the reaction for 15-60 minutes in the dark and measure fluorescence intensity. The end point reading must be done while the enzyme reaction is in the linear phase.

ANALYSIS

Analysis

1. Following assay completion, average the triplicate readings for each sample and each control. To convert RFU to concentration of AMC released, first produce an AMC standard curve as described in the section on page 15.
2. For continuous kinetic analysis:
   a. Plot data as RFU *vs.* time for each sample/control assay.
   b. If the reaction time-course is non-linear, determine the range of initial time points during which the assay response is linear.
   c. Obtain the slope of a line fit to the data, using an appropriate linear regression program, to give the initial reaction velocity (Vo) in RFU/min.
   d. Subtract the reaction rate of the blank control from the rate of all other reactions.
3. For end point analysis:
   a. Subtract the average blank value from each DUB assay data point to give corrected RFU values.
   b. Divide RFU by reaction time to convert to enzyme activity in RFU/min.
4. Perform appropriate data analyses as required, for example Michaelis-Menten kinetics to determine K_M, k_{cat}, and k_{cat}/K_M data.

5. Determining Compound Efficacy:
   a. Plot data as reaction rate *versus* concentration to assess performance of test compounds.
   b. Perform appropriate data analyses as required to determine % inhibition or IC_{50} value.

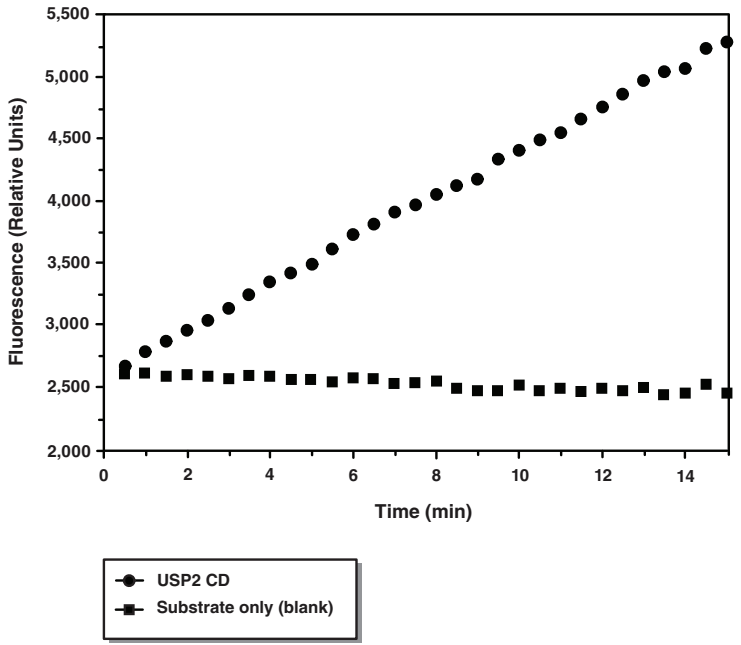

Figure 1. Time course of control DUB enzyme (USP2 CD) activity with Ub-AMC substrate using Cayman's DUB assay kit - Control DUB (10 nM) was incubated with 500 nM Ub-AMC in DUB assay buffer at room temperature alongside a substrate only (blank) control reaction. Fluorescence measurements (RFU) were taken at 30 second intervals and plotted *vs.* time.

AMC standard curve (optional)

Perform an 'in-well' serial dilution of 16 µM AMC Standard Solution in DUB Assay Buffer in triplicate as follows:

- Add 50 µl DUB Assay Buffer to well A
- Add 50 µl DUB Assay Buffer to wells B to H
- Add 50 µl of 16 µM AMC standard working solution to wells A
- Pipette 50 µl from well A and mix it thoroughly with the contents of well B
- Pipette 50 µl from well B and mix it thoroughly with the contents of well C etc. continuing to well G
- Discard the last 50 µl pipetted from well G
- Leave well H as a blank

Well	AMC Standard (nM)
A	8,000
B	4,000
C	2,000
D	1,000
E	500
F	250
G	125
H	0

Table 2. Standard preparation

1. Measure fluorescence reading for AMC standards using microplate reader (360 nm excitation, 460 nm emission).
2. Average the triplicate readings for each AMC concentration.
3. Plot RFU *vs.* AMC concentration to visualize results.
4. Determine slope of the plot using an appropriate linear regression program.

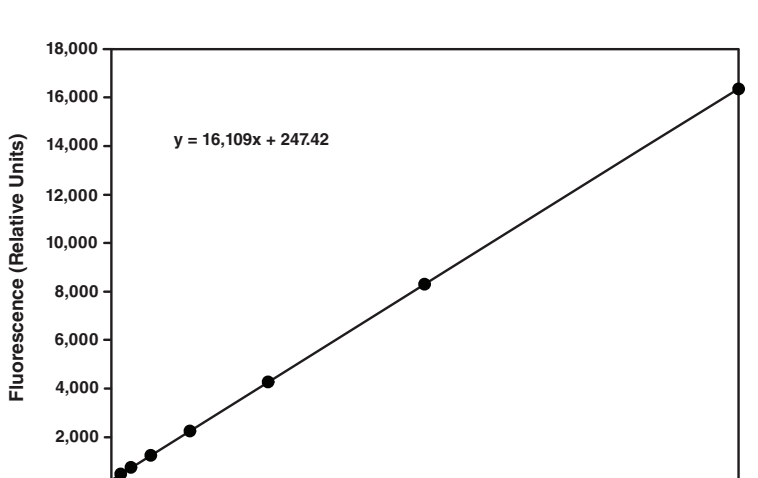

Figure 2. AMC Standard Curve

RESOURCES

References

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Related Products

LC3 Interact Kit - Item No. 15977
Ubiquitin Interact Kit - Item No. 15978
Ubiquitinated Protein Capture Kit - Item No. 15979
UltraPure Water - Item No. 400000
WP1130 - Item No. 15227

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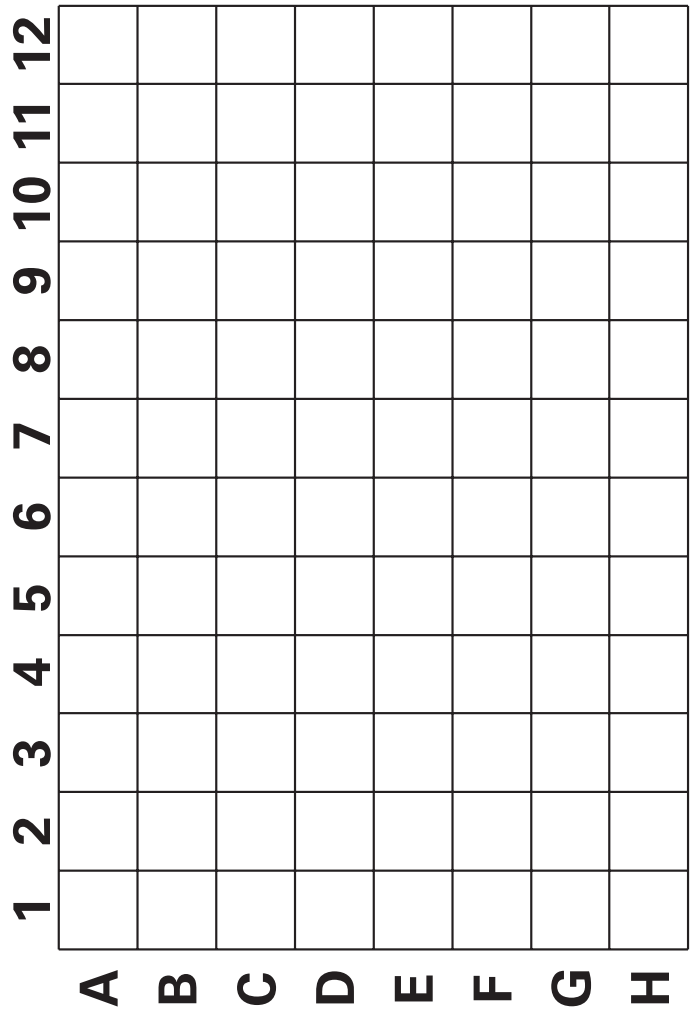
For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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