DUB Activity Assay Kit

Item No. 701490

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION
3 Materials Supplied
4 Safety Data
4 Precautions
4 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION
6 Background
7 About This Assay

PRE-ASSAY PREPARATION
8 Reagent Preparation

ASSAY PROTOCOL
9 Plate Set Up
11 Standard Preparation
12 Performing the Assay

ANALYSIS
13 Calculations
14 Sample Data

RESOURCES
16 References
17 Plate Template
18 Notes
19 Warranty and Limitation of Remedy

GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
701491	DUB Assay Buffer (10X)	1 vial/1 ml	-20°C
701492	Control DUB enzyme (USP2 Catalytic Domain)	1 vial/25 µl	-80°C
701493	Ubiquitin-AMC	1 vial/25 µl	-80°C
701494	AMC Assay Reagent	1 vial/100 µl	-20°C
700416	DTT (1M) Assay Reagent	1 vial/1 ml	-20°C
400012	96-Well Cover Sheet	1 cover	RT
10011288	Half Volume 96-Well Plate (white)	1 plate	RT

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 RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

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

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.

Materials Needed 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. *NOTE: Ultra-Pure water is available for purchase from Cayman (Item No. 400000).*

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.[1] 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.[2] DUBs in general exhibit a wide range of ubiquitination type (mono- or polyubiquitination) and polyubiquitin chain linkage substrate specificities.[3] 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.[1,2]

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. Ubiquitin-AMC is not a suitable substrate for all DUBs. Compatibility must be determined by the end user.

Reagent Preparation

1. **DTT (1M) Assay Reagent - (Item No. 700416)**

   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 - (Item No. 701491)**

   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 (Item No. 700416) 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 - (Item No. 701492)**

   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 - (Item No. 701493)**

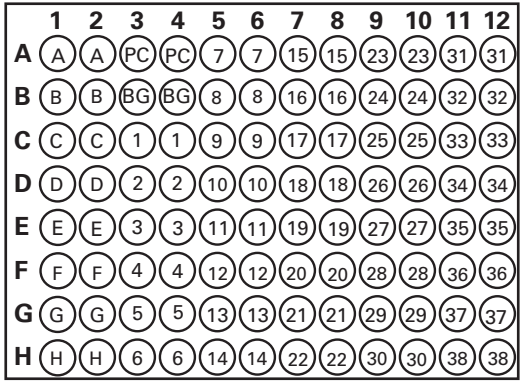
   This vial contains Ubiquitin-AMC in DMSO. Mix 21.8 µl of Ubiquitin-AMC with 478.2 µl 1X DUB Assay Buffer containing DTT. *NOTE: The final concentration of substrate in the assay as described below is 500 nM.*

5. **AMC Assay Reagent - (Item No. 701494)**

   This vial contains 1 mM AMC in DMSO. The reagent is ready to use to prepare the AMC standard curve.

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of samples to be measured in duplicate is shown in Figure 1 below.



A-H = AMC Standards
PC = DUB Positive Control
BG = Background
1-38 = Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
- Avoid introducing bubbles to the well.
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- 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 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.

Standard Preparation

Mix 25 µl of the AMC Assay Reagent (Item No. 701494) 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.

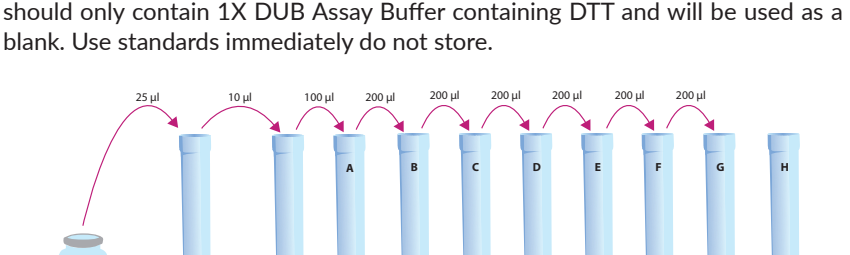


Figure 2. Preparation of the AMC standards

Performing the Assay

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.

Well Type	1X DUB Assay Buffer containing DTT	20 nM DUB Positive Control	Sample
Positive Control Wells	40 µl	5 µl	-
Background Wells	45 µl	-	-
Sample Wells	40 µl	-	5 µl

Table 1. Pipetting Summary

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

Calculations

Plot the Standard Curve

1. Determine the average fluorescence of the standards.
2. Plot the average fluorescence values (from step 1 above) of each standard as a function of the final concentration of AMC from Figure 2, on page 11. See Figure 3 on page 14 for a typical standard curve.

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. An example of USP2 catalytic domain assayed over time is shown in Figure 4 on page 15.

   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:

$$\text{RFU/min} = \frac{\text{RFU (Time 2) - RFU (Time 1)}}{\text{Time 2 (min) - Time 1 (min)}}$$

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.

$$\text{DUB activity (pmol/min/ml)} = \frac{\text{(RFU/min)}}{\text{Standard curve slope (RFU/nM)}} \times \text{Sample dilution}$$

Sample Data

The data shown here is an example of the data typically produced with this kit. Your results may vary and therefore should not be directly compared to these samples.

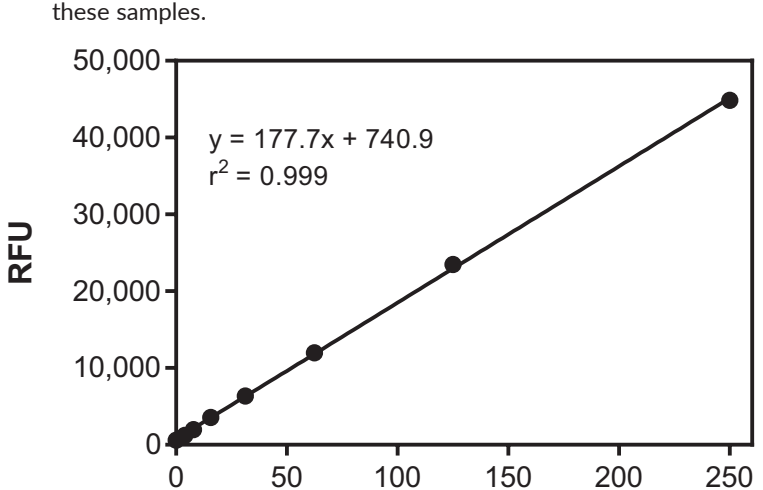


Figure 3. AMC standard curve

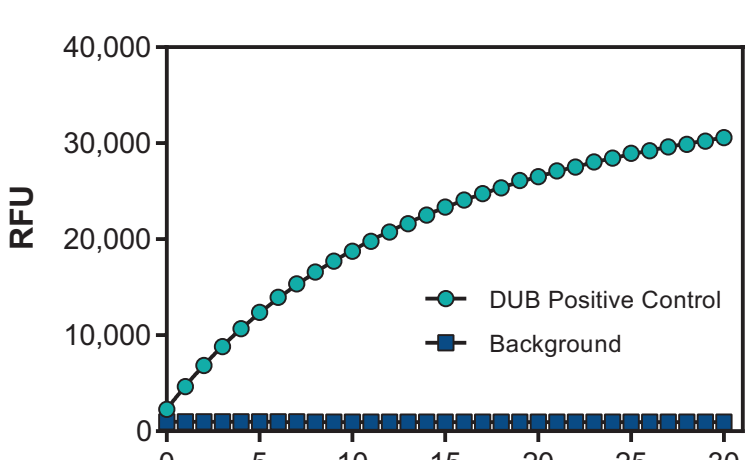
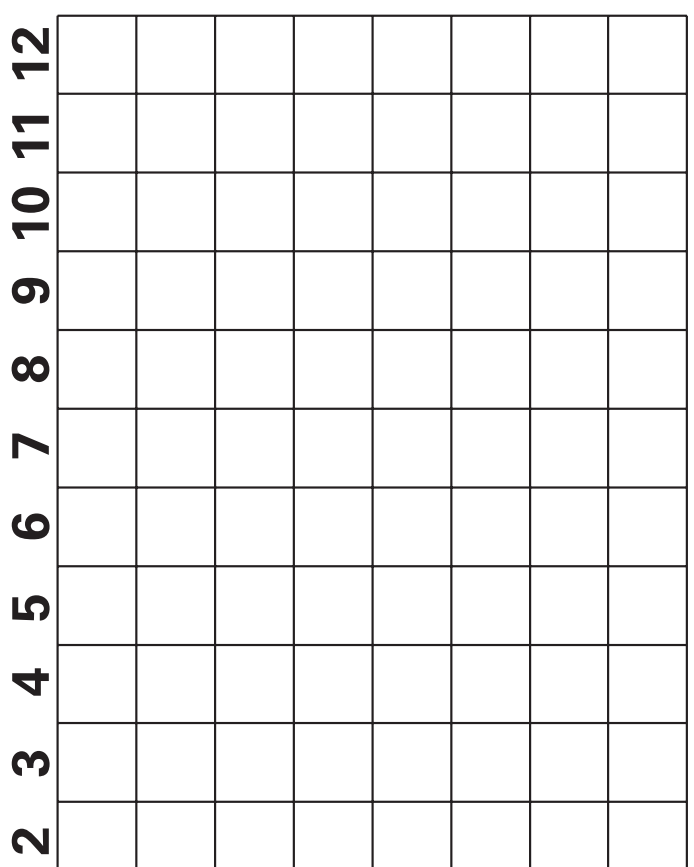


Figure 4. DUB Positive Control Activity

References

1. Fraile, J.M., Quesada, V., Rodrķguez, D., *et al.* Deubiquitinases in cancer: New functions and therapeutic options. *Oncogene* **31(19)**, 2373-2388 (2012).
2. Ciechanover, A. Intracellular protein degradation: From a vague idea through the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Bioorg. Med. Chem.* **21(12)**, 3400-3410 (2013).
3. Komander, D. and Rape, M. The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203-209 (2012).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.