Description

The DUB-Freedom™ Inhibitor Screening Assay Kit is a fluorogenic assay designed to measure the activity of several purified deubiquitinating (DUB) enzymes of interest in a homogeneous 96-reaction format and is ideal for screening and profiling applications. The kit contains enough Ubiquitinated-AMC substrate and assay buffer for 100 reactions. It also includes the purified catalytic domain of the DUB enzyme USP2 (ubiquitin carboxyl-terminal hydrolase 2) (amino acids 259-end) as a positive control and Ub-Aldehyde as control inhibitor.

The enzyme should be preincubated with or without the test inhibitor prior to adding the Ub-AMC substrate to the reaction. The assay was functionally validated using the purified catalytic domain of human USP2 protein and Ub-Aldehyde, a potent inhibitor of the DUB subfamilies Ubiquitin C-terminal Hydrolases (UCHs), Ubiquitin-Specific Proteases (USPs), Ovarian Tumor Proteases (OTU), and Machado-Josephin Domain (MJD) proteases.

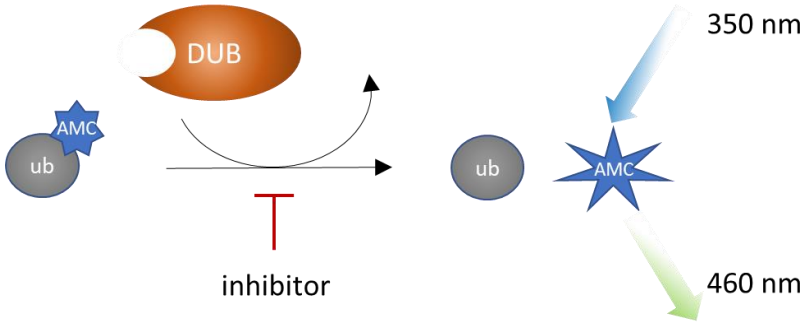

Figure 1: Illustration of the assay principle.

Ubiquitin-AMC (Ub-AMC) is a fluorogenic substrate for ubiquitin hydrolases, based on the C-terminus derivatization of ubiquitin with 7-amido-4-methylcoumarin (AMC). In the conjugated form the energy emitted from AMC is quenched. Upon release from Ub, AMC emissions are no longer quenched and fluorescence with λexcitation/λemission maxima of 350/460 nm is emitted. The increase in fluorescence is proportional to the DUB activity.

Background

Deubiquitinases (DUBs), also known as deubiquitinating peptidases, ubiquitin proteases, ubiquitin hydrolases, ubiquitin isopeptidases, are enzymes that remove ubiquitin or ubiquitin chains from other proteins. These proteins contribute to the ubiquitin signaling pathway by countering the signal induced by ubiquitin ligases and thereby control the stability, subcellular localization and/or interactions of many cellular proteins. DUBs are implicated in many human pathologies including neurodegenerative diseases, cancer, diabetes, and autoimmune pathologies, making them interesting new therapeutic targets.

Applications

- Measure the deubiquitination activity of several DUB enzymes of interest.
- Characterize novel deubiquitinases, or variants of known DUBs.
- Screen inhibitors or activators of a DUB enzyme of interest in high throughput screening (HTS) applications.
- Determine compound IC_{50}.
- Perform DUB real-time analysis.

Supplied Materials

Catalog #	Name	Amount	Storage
80392	USP2, His-Tag (E. coli-derived)*	> 1 µg	-80°C
81150	Ub-AMC Substrate	5 µl	-80°C
79274	10x PR-01 Assay Buffer	3 x 1 ml	-80°C
	Ub-Aldehyde (350 µM)	5 µl	-80°C
	0.5 M DTT	200 µl	-80°C
79685	96-well black microplate	1	Room Temp

** The concentration of protein is lot-specific and will be indicated on the tube containing the protein.*

Materials Required but Not Supplied

- Purified DUB of interest
- Adjustable micropipettor and sterile tips
- Plate reader

Stability

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed.

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Contraindications

- The DUB Freedom™ Inhibitor Screening Assay Kit uses Ub-AMC as a substrate. The user should be aware that while some DUBs are linkage-nonspecific others can discriminate the linkage type, chain length, modifier (Ub or Ubl), and/or the substrate to which Ub is conjugated. Furthermore, reported Ub chain specificities are assay-dependent, and many exceptions exist.
- This kit is compatible with up to 1% final DMSO concentration.

Assay Protocol

- All samples and controls should be performed in duplicate.
- The assay should include a “Negative Control”, two internal controls (“Positive control” and “Inhibitor Control”) and can include the test conditions “DUB Test” and/or “Test Inhibitor”. Additional controls may be appropriate for your experimental design.
- If the assay plate is going to be used more than once, prepare enough of each reagent for this portion of the assay and aliquot the remaining undiluted reagents into single-use aliquots depending on how many times the assay plate will be used. Store the aliquots at -80°C or as recommended for each reagent. Unused diluted proteins should be discarded.

Protect Ub-AMC from direct exposure to light.

1. Prepare 10x PR-01 Assay Buffer with 5 mM DTT by diluting **0.5 M DTT** in **10x PR-01 Assay Buffer**.

2. Prepare a 10-fold dilution of 10x PR-01 Assay Buffer containing 5 mM DTT with distilled water to create 1x Assay Buffer.

3. Thaw **USP2** on ice. Briefly spin the tube to recover its full content.

4. Dilute USP2 to 0.4 ng/µl in Assay Buffer (you need 25 µl/well).

5. Prepare appropriate dilution(s) of the desired DUB enzyme in 1x Assay Buffer (you need 25 µl/well).

   Note: Keep the diluted proteins on ice until use. Do not freeze and re-use the diluted proteins.

6. Add 25 µl of diluted USP2 to the wells designated as "Inhibitor Control" and "Positive Control".

7. Add 25 µl of desired DUB dilution to the "Test DUB" wells.

8. Add 25 µl of 1x Assay Buffer to the "Negative Control" wells.

9. If applicable, prepare the Test inhibitor (5 µl/well): for a titration prepare serial dilutions at concentrations 10-fold higher than the desired final concentrations. The final volume of the reaction is 50 µl.
   9.1. If the Test Inhibitor is water-soluble, prepare serial dilutions in the 1x Assay Buffer at concentrations 10-fold higher than the desired final concentrations. 1x Assay Buffer is the Diluent Solution.

   OR

   9.2. If the Test inhibitor is soluble in DMSO, prepare the test inhibitor in 100% DMSO at a concentration 100-fold higher than the highest desired final concentration, then dilute the inhibitor 10-fold in 1x Assay Buffer to prepare the highest concentration of the serial dilutions. The concentration of DMSO is now 10%.

   Using 1x Assay Buffer containing 10% DMSO, to keep the concentration of DMSO constant, prepare serial dilutions of the Test Inhibitor at 10-fold the desired final concentrations.

   For positive and negative controls, prepare 10% DMSO in 1x Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

   Note: The final concentration of DMSO in the assay should not exceed 1%.

10. If applicable, add 5 µl of Test inhibitor to each well designated "Test Inhibitor".

11. If applicable, add 5 µl of Diluent Solution to the "Positive Control" and "Negative Control" wells.

12. If no inhibitor is being tested add 5 µl of 1x Assay Buffer to "Positive Control" and "Negative Control" wells.

13. Prepare the Inhibitor Control by diluting stepwise Ub-aldehyde (350 mM) in 1x Assay Buffer to make a 10 µM solution.

14. Add 5 µl of diluted Ub-aldehyde to the "Inhibitor Control" wells (final concentration will be 1 µM).

15. If applicable, preincubate the Test inhibitor and the DUB for 30 minutes at Room Temperature (RT) with gentle agitation.

16. Dilute Ub-AMC Substrate 400-fold with 1x Assay Buffer.

17. Initiate the reaction by adding 20 µl of diluted Ub-AMC Substrate to all wells. **Protect your samples from direct exposure to light.**

18. Incubate the plate at RT for 30 minutes or perform kinetic analysis.

	Negative Control	**Positive Control**	**Inhibitor Control**	**Test DUB**	**Test Inhibitor**
1x Assay Buffer	25 µl	-	-	-	-
Test Inhibitor (if applicable)	-	-	-	-	5 µl
Ub-Aldehyde	-	-	5 µl	-	-
Diluent Solution	5 µl	5 µl	-	5 µl	-
Diluted USP2 (0.4 ng/µl)	-	25 µl	25 µl	-	-
Test DUB	-	-	-	25 µl	25 µl
Ub-AMC Substrate	20 µl	20 µl	20 µl	20 µl	20 µl
Total	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**

19. Read the fluorescence intensity of the samples (lexcitation = 350 nm; lemission = 460 nm) in a fluorescence reader.

Example Results

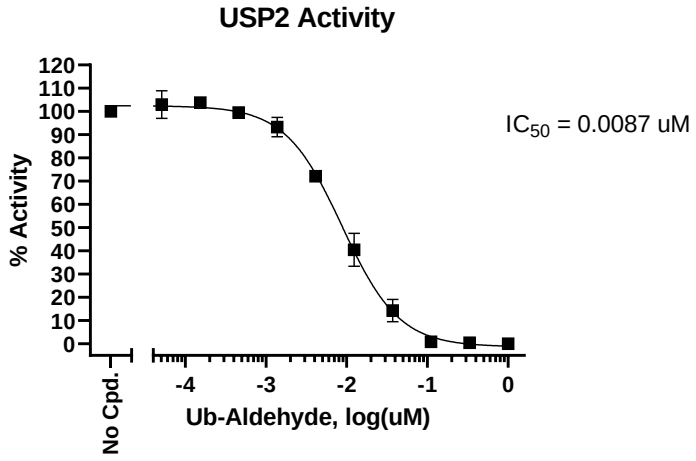

Figure 2. USP2 inhibition by Ub-Aldehyde.
USP2 activity was measured in the presence of increasing concentrations of Ub-Aldehyde. Results are expressed as percent activity, in which the activity of USP2 in absence of inhibitor is set to 100%.

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com

Troubleshooting Guide
Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For further questions, please email support@bpsbioscience.com

Related Products

Products	*Catalog #*	*Size*
USP2 Inhibitor Screening Assay Kit	78859	96 reactions
USP2, FLAG-Tag (SF9-derived) Recombinant	80352	50 µg
UCHL3 Inhibitor Screening Assay Kit	78815	96 reactions
USP14 Inhibitor Screening Assay Kit	78865	96 reactions