StressMarq Biosciences INC.

Discovery through partnership | Excellence through quality

StressXpress®

DUB Activity Kit

Catalog# SKT-136 (96-Well Kit)

Fluorometric detection of DUB activity

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GENERAL INFORMATION

Materials Supplied

Catalog Number	Reagent	Quantity	Concentration
SKC-136A	10x StressXpress® DUB assay buffer	1mL	10x
SKC-136B	Control DUB enzyme (USP2 catalytic domain)	10µL	10µM
SKC-136C	Ubiquitin-AMC	25µL	1mg/mL
SKC-136D	StressXpress® 96 well assay plate	1 plate	N/A
SKC-136E	StressXpress® AMC standard	17.5µg	Solid

If any of the items listed above are damaged or missing, please contact our Customer Service department at (250) 294-9065. We cannot accept any returns without prior authorization.

WARNING: Not for human or animal disease diagnosis or therapeutic drug use.

Precautions

Please read these instructions carefully before beginning this assay.

Kit contains sufficient materials for one 96-well plate assay, controls and optional calibration standards. The reagents in this kit have been tested and formulated to work exclusively with StressMarq Biosciences Inc.'s StressXpress® Kits. This kit may not perform as described if any reagent or procedure is replaced or modified.

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

If You Have Problems

Technical Service Contact Information

Phone:	250-294-9065
Fax:	250-294-9025
E-Mail:	techsupport@stressmarq.com
Hours:	M-F 9:00 AM to 5:00 PM PST

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

The StressXpress® DUB assay kit should be stored at -80ºC to ensure stability and activity. StressXpress® 96 well assay plate can be stored at ambient temperature. Once dissolved AMC standard solutions can be aliquoted and stored at -80ºC for up to 3 months protected from light. For storage of kit component working solutions see 'Assay preparation'. Avoid multiple freeze-thaws.

Materials Needed But Not Supplied

- Fluorescence microplate reader with filters for 360nm excitation and 460nm emission
- Microfuge tubes (1.5mL)
- Sterile water (dH_2O)
- Dithiothreitol (DTT) – prepare solutions fresh
- 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, amongst others, 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 substrate, ubiquitination type (mono- or poly-ubiquitination) and polyubiquitin chain linkage specificities and 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

A rapid, robust, easy-to-use kit for measurement of DUB activity

- Measure activity of your deubiquitinating enzyme in continuous kinetic and end-point assays
- Utilise industry standard fluorogenic substrate ubiquitin-AMC for sensitive analysis of DUB activity
- Test DUB enzymes, activators and inhibitors in 96-well plate HTS compatible format
- Quantify results using calibration standards supplied

The StressXpress® DUB assay kit facilitates the rapid, robust measurement of deubiquitinating enzyme activity in vitro. The kit utilises 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 96-well plate format for multi-sample analysis. Contains sufficient materials for one full 96-well plate assay set-up to be run.

Use this kit 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. Optimise assays for specific DUBs to facilitate their use in high throughput screening (HTS)
5. Demonstrate novel, putative DUB enzymes have ubiquitin-AMC processing activity

PRE-ASSAY PREPARATION

Overview

The StressXpress® DUB assay protocol described can be utilised or adapted to:

- Assess performance of DUB enzymes, activators and inhibitors of interest enabling relevant kinetic data to be generated
- Optimise assays for specific DUBs to facilitate their use in high throughput screening (HTS)
- Demonstrate novel, putative DUB enzymes for ubiquitin-AMC processing activity
- Screen potential inhibitors and activators for activity against specific DUB enzymes

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

Assay Preparation

DUB assay buffer

Dilute 10× StressXpress® DUB assay buffer supplied 10 fold with dH_2O as required to give 1× DUB assay buffer for use in assays and preparation of diluted solutions.

Storage: 1× DUB assay buffer can be stored short term at 4°C and longer term at -20°C or -80°C

Ubiquitin-AMC (Ub-AMC)

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

- Ub-AMC substrate supplied at 1mg/mL in DMSO (114.4µM)
- Prepare a 5µM working solution by 22.9× dilution in 1× DUB assay buffer
- Add 21.8µL stock Ub-AMC to 478.2µL 1× DUB assay buffer to give 500mL 5µM Ub-AMC– sufficient for ~100 DUB assays at 500nM Ub-AMC assay concentration

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

Control DUB enzyme (USP2 CD)

- Recommend 10nM control DUB enzyme assay concentration
- USP2 CD supplied at 10µM in 1× DUB assay buffer
- Prepare a 100nM working solution by 100× dilution in 1× DUB assay buffer
- Add 10µL 10µM USP2 CD to 990µL 1× DUB assay buffer to give 1mL 100nM control DUB enzyme solution

Storage: 10µM control DUB enzyme solution can be stored long term at-80°C. The 100nM control DUB enzyme solution can be stored for up to 3 months at -80°C.

Sample DUB enzyme

- Recommend 10nM sample DUB enzyme assay concentration as a starting point.
- Optimal assay concentration for specific DUB enzymes will need to be determined by the user
- Prepare appropriate sample DUB enzyme working solution concentrations by dilution in 1× DUB assay buffer, for example at 100nM working solution for 10× dilution to 10nM assay concentration.

AMC standard (optional)

- AMC standard supplied as a solid (17.5µg)
- Prepare a 100µM AMC standard stock solution by dissolving solid in 100µL DMSO, transfer solution to a larger tube and addition of 900µL DMSO
- Prepare a 10µM AMC standard working solution by 10× dilution in 1× DUB assay buffer
- Add 10µL 100µM AMC standard stock solution to 90µL 1× DUB assay buffer to give 100µL working solution

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

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 optimised using the continuous analysis approach prior to application in end point assay set-ups.

DUB activation by DTT

1× DUB assay buffer contains 1mM DTT however some DUBs may require DTT activation prior to use. In this case inclusion of DTT to a final concentration of 10mM in 1× DUB assay buffer is recommended followed by the standard 15-30 minutes incubation of assays 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 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 – adjust readings for background intrinsic substrate fluorescence and any auto-hydrolysis of Ub-AMC
- **Positive control:** USP2 cd – demonstrate 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) – determine effect, if any, of carrier for test compounds on assay performance

Assay optimisation

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

ASSAY PROTOCOL

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

1. Set-up the microplate reader for recording at 360nm excitation and 460nm emission.
2. Prepare the required number of sample/control DUB assays (50µL final volume) in an appropriate number of wells by addition of assay components in 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 plate at room temperature for 15-30 minutes
5. Add Ub-AMC to initiate assays and mix reagents by shaking plate gently for 15 seconds

Component	Sample DUB enzyme	Control DUB enzyme	Substrate only (blank)
1× DUB assay buffer*	40.0	40.0	45.0
100nM sample DUB enzyme	5.0	-	-
100nM USP2 CD	-	5.0	-
	Incubate for 15-30 minutes at room temperature		
5µM Ubiquitin-AMC	5.0	5.0	5.0

*Adjust DUB assay buffer volume to allow for DUB enzyme, Ubiquitin-AMC and activator / inhibitor volume used

6. Measure the fluorescence signal:

a. F**or continuous kinetic reading:** Commence timecourse assay data acquisition immediately upon addition of Ub-AMC substrate using microplate reader. Monitor RFU response for 15-60 minutes taking regular readings (see Figure 1 for USP2 CD example assay)

b. F**or end point reading:** Incubate the reaction for 15-60 minutes in the dark. Mix the reagents and measure fluorescence intensity using microplate reader

Data Analysis

1. Following assay completion average the triplicate readings for each sample, control and substrate only (blank) assay and subtract the average blank value from each DUB assay data point to give RFU values
2. **For continuous kinetic analysis:**
   a. Plot data as RFU vs. time for each sample / control assay. To convert RFU to concentration of AMC released first produce an AMC standard curve, as described in the following section
   b. If 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. Perform appropriate data analyses as required, for example Michaelis-Menten kinetics to determine K_M, k_{cat} and kcat/K_M data
3. **For end point analysis:**
   a. Plot data as RFU versus concentration, for example to assess performance of test compounds.
   b. Perform appropriate data analyses as required, for example determining % inhibition % or IC_{50}.

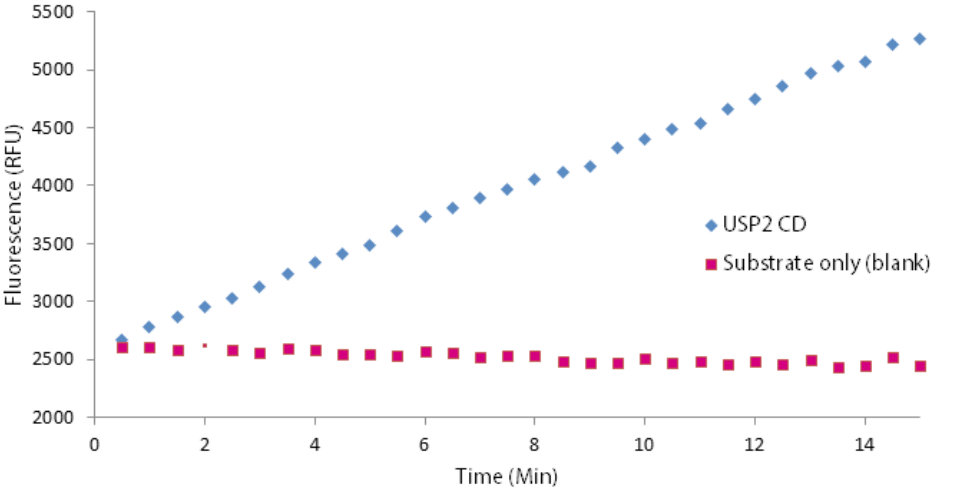

Figure 1: Time course of control DUB enzyme (USP2 CD) activity with Ub-AMC substrate using StressXpress® DUB assay kit – Control DUB (10nM) was incubated with 500nM Ub-AMC in DUB assay buffer for 15 minutes at room temperature alongside a substrate only (blank) control reaction. Fluorescence measurements (RFU) were taken at 30 second intervals and plotted vs. time

AMC standard curve (optional)

Perform an 'in-well' serial dilution of 10µM AMC Standard Solution in DUB Assay Buffer **in triplicate** as follows:

- Add 90µL DUB Assay Buffer to well A
- Add 10µL 10µM AMC standard working solution to wells A
- Add 50µL DUB Assay Buffer to wells B to H
- Pipette 50µL from well A and mix it thoroughly with the contents of well B
- Pipette 50µL from well B and mix it thoroughly with the contents of well C etc. up to well G
- Discard the last 50µL pipetted from well G
- Leave well H as a blank

	AMC Standard (nM)		
	1	2	3
A	1000	1000	1000
B	500	500	500
C	250	250	250
D	125	125	125
E	62.5	62.5	62.5
F	31.25	31.25	31.25
G	15.625	15.625	15.625
H	0.0	0.0	0.0

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

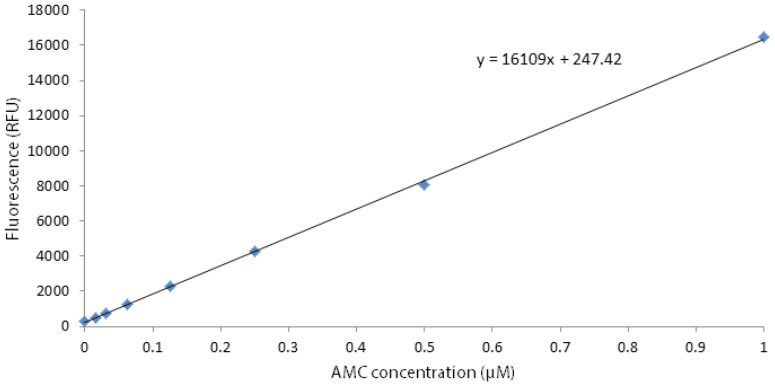

Figure 2: AMC Standard Curve – representative plot of fluorescence measurements (RFU) of serial dilutions of AMC Standard at recommended concentrations.

Plate Configuration Table

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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Buyer's **exclusive remedy** and StressMarq's sole liability hereunder shall be limited to a refund of the purchase price, or at StressMarq's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to StressMarq within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Refund Policy located on our website and in our catalog.

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