StressMarq Biosciences INC.

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StressXpress®

20S Proteasome Activity Kit GOLD

Catalog# SKT-134 (96-Well Kit)

Fluorometric detection of purified 20S proteasome activity

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GENERAL INFORMATION

Materials Supplied

Catalog Number	Reagent	Quantity
SKC-134A	10x StressXpress® proteasome assay buffer	2mL
SKC-134B	20S proteasome	20µg
SKC-134C	MG132 (control inhibitor)	100µg
SKC-134D	StressXpress® 96 well assay plate	2 plate
SKC-134E	StressXpress® AMC standard	1.75µg
SKC-134F	Suc-LLVY-AMC (chymotrypsin-like substrate)	500µg
SKC-134G	Bz-VGR-AMC (trypsin-like substrate)	500µg
SKC-134H	Z-LLE-AMC (caspase-like substrate)	500µg

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 or up to 200 x 50µL micro-cuvette based assays, 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® 20S proteasome assay kit can be stored as supplied at 4ºC for up to 3 months, until ready to use, to ensure stability and activity. For storage of kit component solutions see 'Assay preparation'.

Materials Needed But Not Supplied

- Fluorescence microplate reader with filters for 360nm excitation and 460nm emission
- Microfuge tubes (1.5mL)
- Sterile water (dH_2O)
- Dimethyl sulfoxide (DMSO)

INTRODUCTION

Background

Proteasomes are non-lysosomal proteolytic complexes localised primarily in the cytoplasm and in the nucleus of eukaryotic cells. They are responsible for the ubiquitin-mediated degradation of short half-life proteins and peptides that are involved in essential cellular processes including cell-cycle regulation, apoptosis and transcriptional regulation, innate immunity and antigen processing, and in the removal of redundant or damaged proteins. As such protein degradation by the ubiquitin-proteasome pathway has a major regulatory function for proliferation activity and survival of both normal and malignant cells, and its dysfunction has been implicated in a wide range of other disease processes including neurodegenerative, cardiovascular and metabolic disorders.

The 26S proteasome structure is composed of a 20S proteasome catalytic core complex and one or two 19S (PA700) regulatory subcomplexes. The 20S core comprises two copies of 14 subunits (7 alpha subunits and 7 beta subunits) arranged in a α7β7β7α7 cylindrical array. Proteolytic activities are determined by the β1 (caspase-like), β2 (trypsin-like) and β5 (chymotrypsin-like) subunits, access to which is guarded by the α-subunits. The 19S regulatory unit consists of six ATPase and at least ten non-ATPase subunits that are required for ubiquitinated protein binding, deubiquitination, substrate unfolding and translocation to the 20S catalytic core.

Varying catalytic subunit composition (β1, β1i; β2, β2i; β5, β5i) results in a variety of possible subtypes from full constitutive proteasome (β1, β2, β5) through mixed populations to full inducible / immunoproteasome (β1i, β2i, β5i). Alternative regulatory complexes such as the PA200 and 11S proteasome activators confer different substrate specificities and activity compared to the 19S regulator.

About This Assay

Flexible, robust, easy-to-use kit for measurement of all three activities of purified 20S proteasomes

- Measure purified 20S proteasome activities in continuous kinetic and end-point assays
- Utilise high sensitivity fluorogenic substrates for analysis of proteasome chymotrypsin-like (β5), trypsin-like (β2) and caspase-like (β1) activities
- Test 20S proteasome activators and inhibitors in micro-cuvette or 96-well plate HTS compatible format
- Quantify results using calibration standards supplied

The StressXpress® 20S proteasome assay kit GOLD facilitates the rapid, robust measurement of purified proteasome activity. The kit utilises high purity, fluorogenic substrates Suc-LLVY-AMC, Bz-VGR-AMC, and Z-LLE-AMC together with suitable calibration standards and controls for the accurate and sensitive assessment of proteasome chymotrypsin-like (β5), trypsin-like (β2) and caspase-like (β1) activities. Continuous kinetic or end-point assays can be performed in micro-cuvettes or in 96-well plate format for multi-sample analysis. Contains sufficient materials for two full 96-well plate assays or up to 200x 50µL micro-cuvette based assays to be run.

Use this kit to

1. Screen potential proteasome inhibitors and activators – determine effect on each individual proteasome catalytic activity
2. Assess proteasome chymotrypsin-like (β5), trypsin-like (β2) and caspase-like (β1) activities allowing relevant kinetic data (e.g. K_M, k_{cat}, k_{cat}/K_M) or specific activities to be calculated
3. Evaluate performance and activity-type specificity of proteasome activators and inhibitors enabling relevant kinetic data to be determined (e.g. IC_{50} / EC_{50}, % inhibition / activation, K_i)
4. Investigate effect of activator complexes and binding proteins on proteasome activity
5. Optimise assays for specific user provided purified proteasome preparations to facilitate their use in high throughput screening (HTS)

PRE-ASSAY PREPARATION

Overview

The StressXpress® 20S proteasome assay GOLD protocol described can be utilised or adapted to:

- Assess performance and activity-type specificity of 20S proteasomes, activators and inhibitors of interest enabling relevant kinetic data to be generated
- Screen potential proteasome inhibitors and activators– determine effect on each individual proteasome catalytic activity
- Investigate effect of activator complexes and binding proteins on proteasome activity
- Run assays in micro-cuvette based format
- Optimise assays for specific user provided proteasome preparations to facilitate their use in high throughput screening (HTS)

Assay Preparation

Proteasome assay buffer

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

Storage: Proteasome assay buffer solutions can be stored short term at 4°C and longer term at -20°C or -80°C.

Substrates (Suc-LLVY-AMC, Bz-VGR-AMC, Z-LLE-AMC)

Recommend 100µM substrate assay concentration. All substrates (500µg) supplied lyophilised. Prepare a 10mM stock solutions by dissolving substrates in DMSO as follows:

Substrate	Activity	DMSO
Suc-LLVY-AMC (SKC-134F)	Chymotrypsin-like (β5)	65.4µL
Bz-VGR-AMC (SKC-134G)	Trypsin-like (β2)	84.5µL
Z-LLE-AMC (SKC-134H)	Caspase-like (β1)	75.2µL

Prepare a 1mM substrate working solution by 10× dilution in 1× proteasome assay buffer – add 10µL 10mM substrate stock solution to 90µL 1× proteasome assay buffer to give 100µL working solution.

Storage: Stock and working solutions can be stored at -80ºC for up to three months protected from light. Avoid multiple freeze-thaws.

20S proteasome

Recommend ~2.5nM 20S proteasome assay concentration (0.1µg / 50µL reaction)

- 20S proteasome supplied at 1mg/mL in 1× proteasome assay buffer
- Prepare the required amount of 20µg/mL (~25nM) 20S proteasome by 50× dilution with 1× proteasome assay buffer – for example, add 10µL 1mg/mL 20S proteasome to 490µL 1× proteasome assay buffer to give 0.5mL 20µg/mL 20S proteasome solution (5µL required per reaction)

Storage: DILUTED 20S PROTEASOME SOLUTION MUST BE PREPARED AND USED FRESH – DO NOT STORE. 20S proteasome may be stored as supplied for up to six months at 4ºC. Recommend storage at 4ºC if material is to be used on a regular basis. For longer term storage prepare single use aliquots, add high purity glycerol to 50% and store at -80ºC. Avoid freeze-thaw cycles. Use frozen 20S proteasome aliquots immediately upon thawing to ensure activity retained.

NOTE: USER PREPARED 20S PROTEASOME CAN BE ANALYSED USING THE STRESSXPRESS® 20S PROTEASOME ASSAY KIT BY DILUTING IN 1× PROTEASOME ASSAY BUFFER AND USING 2.5NM ASSAY CONCENTRATION AS A STARTING POINT

MG132 (control inhibitor)

Recommend 5µM MG132 inhibitor assay concentration

- MG132 (100µg) supplied lyophilised
- Prepare a 500µM stock solution by dissolving solid in 420µL DMSO
- Prepare a 50µM working solution by 10× dilution in 1× proteasome assay buffer – add 10µL 500µM MG132 stock solution to 90µL 1× proteasome assay buffer to give 100µL working solution

Storage: Stock and working solutions can be stored at -80ºC for up to one month

AMC standard (optional)

AMC standard supplied as a solid (1.75µg)

- Prepare a 160µM AMC standard stock solution by dissolving solid in 62.5µL DMSO
- Prepare a 16µM AMC standard working solution by 10× dilution in 1× proteasome assay buffer – add 10µL 160µM AMC standard stock solution to 90µL 1× proteasome assay buffer to give 100µL standard working solution

Storage: AMC standard solutions can be stored at -80ºC for up to three months protected from light. Avoid multiple freeze-thaws

End point assays (linear response)

In order to run meaningful end point assays, for example to screen for proteasome activators and inhibitors, the assay conditions used must give a linear response (RFU vs. time) over the course of the assay. When using the StressXpress® 20S proteasome assay kit GOLD for the first time it is recommended that linear response for the starting point assay set-up given is verified for your system using the continuous analysis approach prior to application in end point assays

Proteasome activation by SDS

It has often been reported that SDS can be used to activate the 20S core proteasome. This should be treated with caution as results, upon attempted 20S proteasome activation by SDS, are at best mixed and can sometimes lead to inhibition of activity

Activators / inhibitors

Pre-incubate at desired concentration with proteasome in assay mixture at assay temperature for 15-30 minutes prior to initiation by addition of substrate.

AMC standard curve (optional)

Plotting an AMC standard curve allows proteasome 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

Recommended: Substrate only control: No 20S proteasome – adjust readings for background intrinsic substrate fluorescence and any auto-hydrolysis of substrates. Positive control: 20S proteasome – demonstrate assay working / components active.

Optional: Inhibitor control: Test proteasome inhibition with MG132 control inhibitor – comparison for inhibitor assays. 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 experiments must be determined by the user. Adjustment of the following parameters may facilitate this process:

- 20S proteasome concentration, 2.5-20nM
- Suc-LLVY-AMC concentration, 50-500µM
- Assay incubation (end point) or monitoring (continuous) time, 15-60 minutes
- Assay temperature, increase from room temperature to 30-37°C
- Activator / inhibitor concentration and pre-incubation time

ASSAY PROTOCOL

Key points for assay protocol:

- Protocol described for 96-well plate format, assays can be run and analysed in micro-cuvette format if required (up to 200x 50µL assays)
- Recommended that all samples, controls and standards are run in duplicate
- Ensure substrate solutions 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 20S proteasome 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 substrates to initiate assays and mix reagents by shaking plate gently for 15 seconds (refer to table)
6. Measure the fluorescence signal:

a. **For continuous kinetic reading:** Commence timecourse assay data acquisition IMMEDIATELY upon addition of Suc-LLVY-AMC substrate using microplate reader. Monitor RFU response for 15-60 minutes taking regular readings (see Figure 1 for control 20S proteasome assays)

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

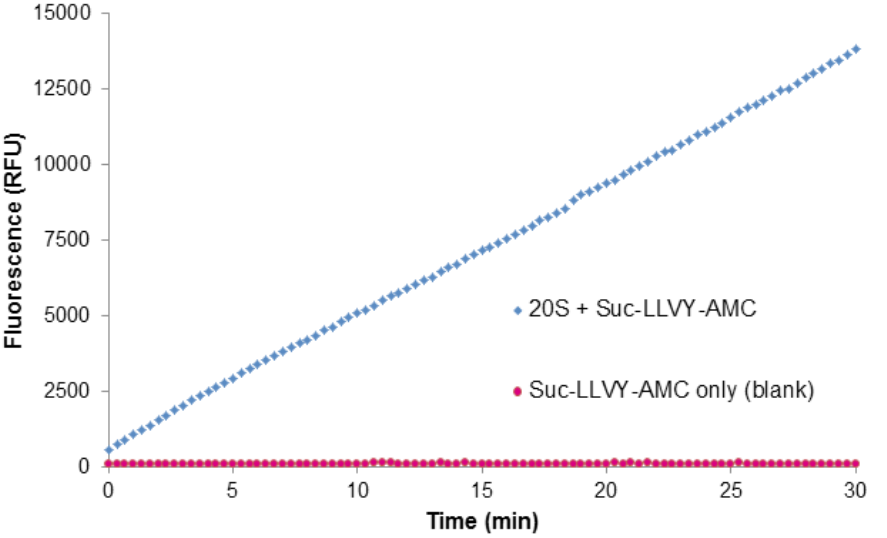
	Suc-LLVY-AMC (chymotrypsin-like)			Bz-VGR-AMC (trypsin-like)			Z-LLE-AMC (caspase-like)		
Component	**20S proteasome**	**Substrate only (blank)**	**Control inhibitor**	**20S proteasome**	**Substrate only (blank)**	**Control inhibitor**	**20S proteasome**	**Substrate only (blank)**	**Control inhibitor**
1× proteasome assay buffer*	40.0	45.0	35.0	40.0	45.0	35.0	40.0	45.0	35.0
25nM 20S proteasome	5.0	-	5.0	5.0	-	5.0	5.0	-	5.0
50µM MG132 (control inhibitor)	-	-	5.0	-	-	5.0	-	-	5.0
Incubate for 15-30 minutes at room temperature									
1mM Suc-LLVY-AMC	5.0	5.0	5.0	-	-	-	-	-	-
1mM Bz-VGR-AMC	-	-	-	5.0	5.0	5.0	-	-	-
1mM Z-LLE-AMC	-	-	-	-	-	-	5.0	5.0	5.0

*Adjust proteasome assay buffer volume to allow for 20S proteasome, substrate and activator / inhibitor volume used

Data Analysis

1. Following assay completion average the duplicate readings for each sample, control and substrate only (blank) assay and subtract the average blank value from each 20S proteasome 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 k_{cat}/K_M data or to calculate specific activity
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}

A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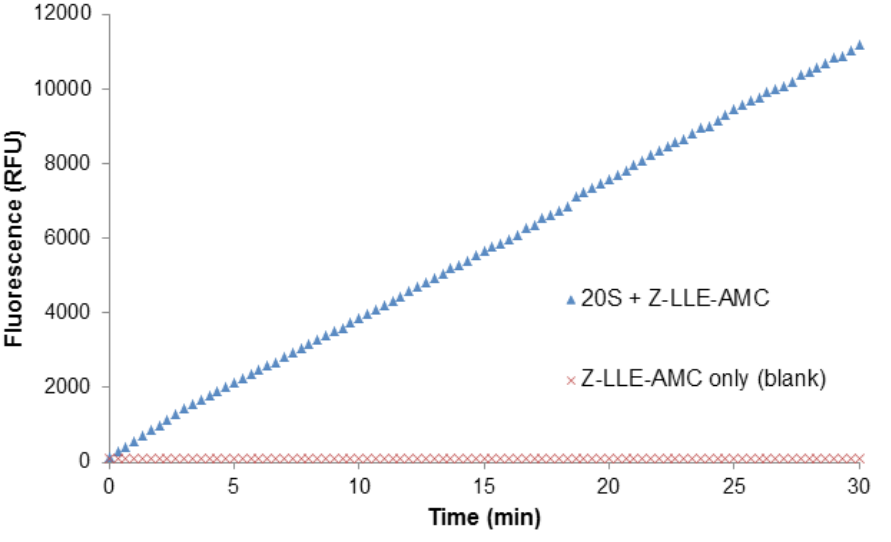
Time (min)
20S + Suc-LLVY-AMC
Suc-LLVY-AMC only (blank)

B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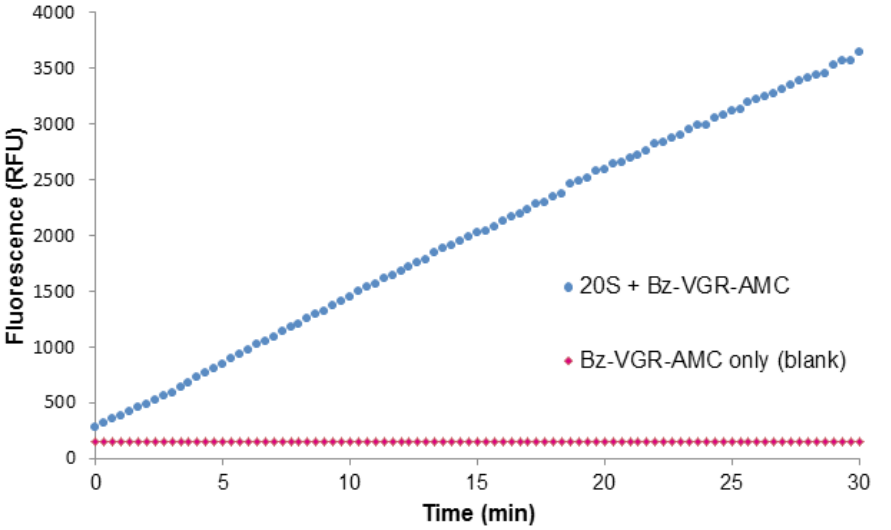
Z-LLE-AMC only (blank)

C



A-C: Time course of control 20S proteasome activity with A) Suc-LLVY-AMC, B) Z-LLE-AMC and C) Bz-VGR-AMC using the StressXpress® 20S proteasome assay kit GOLD – In each case 20S proteasome (~2.5nM) was incubated with 100µM substrate in proteasome assay buffer for 30 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 16µM AMC standard working solution in proteasome assay buffer **in duplicate** as follows:

- Add 50µL proteasome assay buffer to wells A to H
- Add 50µL 16µM AMC standard working solution to well A
- 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 (µM)	
	1	2
A	8.0	8.0
B	4.0	4.0
C	2.0	2.0
D	1.0	1.0
E	0.5	0.5
F	0.25	0.25
G	0.125	0.125
H	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

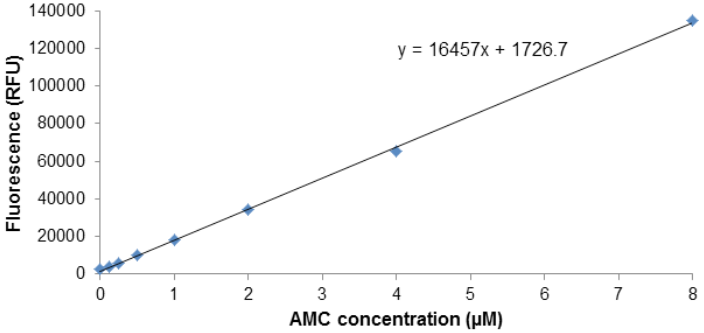


Figure 2: AMC Standard Curve. Representative plot of fluorescence measurements (RFU) of serial dilutions of AMC Standard at recommended concentrations.oncentrations

Plate Configuration Table

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

References

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Warranty and Limitation of Remedy

StressMarq Biosciences Inc. makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. StressMarq **warrants only** to the original customer that the material will meet our specifications at the time of delivery. StressMarq will carry out its delivery obligations with due care and skill. Thus, in no event will StressMarq have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if StressMarq is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of StressMarq, its directors or its employees.

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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