20S Proteasome Assay Kit (SDS Activation Format)

Catalog Number K-900

This kit contains buffers and reagents for the quantitative analysis of 20S proteasome activity in cuvettes or a 96-well microtiter plate formats. The 20S activity is measured by monitoring the release of free AMC from the fluorogenic peptide suc-LLVY-AMC (S-280).

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The Ubiquitin Proteasome System (UPS) is the cell's principle mechanism for protein catabolism. The UPP has been shown to have significant involvement in the regulation of critical cellular processes such as transcription, oncogenesis, cell cycle progression, development, growth, selective elimination of abnormal proteins, and antigen processing (1-3). The proteasome is a large, multimeric protease that catalyzes the final step of the UPS intracellular protein degradation. The proteasome exists in multiple forms within the eukaryotic cell, and contained in all isoforms is the catalytic core known as the 20S proteasome. The 20S proteasome (700 kDa) is arranged as four axially stacked heptameric rings with two β-subunit rings sandwiched between two α-subunit rings. The multicatalytic centers are located within the internal cavity of the β-subunits. The 20S proteasome is characterized by three distinct proteolytic activities against short synthetic peptides: chymotryptic-like (Tyr or Phe at P1), tryptic-like (Arg or Lys at P1) and peptidylglutamyl peptide- hydrolyzing (Glu at P1) (4). These activities are believed to be catalyzed by the nucleophilic N-terminal threonines of the β-subunits (5, 6). The 20S proteasome *in vitro* cannot efficiently degrade peptides or proteins unless they are highly denatured and the 20S has been activated by the addition of low concentrations of sodium dodecyl sulfate (SDS) or PA28 (or 11 S activator) (7-12). The exact mechanism for this apparent SDS activation is not well-understood, except to postulate that SDS induces conformational changes under limited denaturation thereby allowing access to the central cavity where the active sites residues reside.

The most common assessment of 20S activity *in vitro* is done by measuring the hydrolysis of the fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-AMC (suc-LLVY-AMC, Catalog # S-280) by the SDS-activated proteasome. This substrate is cleaved by the chymotryptic-like activity of the proteasome releasing free AMC (7-amino-4-methylcoumarin) which can be efficiently detected using a fluorimeter with excitation and emission wavelengths of 345 nm and 445 nm, respectively. Purified 20S proteasome chymotryptic-like activity is dependent on SDS concentration up to 0.030% SDS where the activity is maximal (Fig.1). SDS concentrations beyond 0.03% result in inactivation due to excessive and irreversible denaturation of the 20S. When this substrate is titrated against a fixed concentration of proteasome pre-activated with SDS, Suc-LLVY-AMC exhibits a KM value of 9 μM (Fig. 2). At saturating substrate concentrations (≥50 μM), the turn-over rate (V_{max}) is approximately 12 s^{-1}.

TECHNICAL HINTS

- For quantitative measurements of proteasome activity using the AMC-based substrates, the fluorimeter may be calibrated by generating a standard curve using AMC (available separately). The standard curve should have a concentration range of 0-800 nM AMC. This calibration allows for the calculation of specific activity of the 20S on each individual fluorimeter.
- For optimum activity, it is recommended that the assay be performed at 37°C. However, if the measurement instrument has no temperature control, the assay may be performed at room temperature. The activity signal at 25°C will decrease approximately 3-4 fold relative to signal measured at 37°C.
- It is important to prepare all solutions by thawing them briefly in a warm water bath. A quick centrifugation is recommended to limit loss of materials on tube sides and caps.
- Prior to use, warm and briefly vortex the SDS solution to ensure that it is in solution.
- Depending on what purpose this kit is being used for, the addition order of substrate, SDS, and 20S may vary. Components should be diluted into reaction buffer and mixed well prior to the addition of other kit reagents.

Measurement	Order of Addition into Cuvette or Plate
20S Activity/Inhibition	Buffer, SDS, 20S, Substrate, Inhibitor/Agent
20S Inhibition (with pre-incubation)	Buffer, SDS, 20S, Inhibitor, Substrate
20S SDS Activation	Buffer, 20S, Substrate, SDS
20S Latent Activity (non-activated)	Buffer, 20S, Substrate

- Many types of agents may be tested in the assay. Although the concentration of SDS for maximal activation (0.03%) is below the critical micellar concentration (CMC), SDS still may interact with some agents (*i.e.* unstable proteins and highly positively charged agents). In such cases, an alternate method of activating the 20S proteasome is activator PA28 (Catalog # E-380 or E-381).
- Due to the inherent decreased sensitivity of fluorimetric plate readers compared to cuvette-based fluorimeters, higher enzyme concentrations or longer incubations with substrate may be needed to produce a strong signal when using the kit in microtiter plate format.

MATERIALS PROVIDED

Kit contains buffers and reagents for the quantitative analysis of 20S proteasome activity in cuvettes or a 96-well microtiter plate formats.

COMPONENT	VOLUME	DESCRIPTION
Enzyme Solution, 20S Proteasome	25 µg	1.4 mg/ml (2 µM) Buffer: 50 mM HEPES, pH 8.0, 1 mM DTT
20Z Reaction Buffer	2 x 1.5 mL	500 mM HEPES, 10 mM EDTA, pH 7.6
100X Activation Solution	250 µL	3% SDS (w/v) in dH_20
1000X Substrate Solution	50 µL	10 mM Suc-LLVY-AMC in DMSO

STORAGE CONDITIONS

COMPONENT	STORAGE OF UNOPENED COMPONENTS	STORAGE OF THAWED/ACTIVATED COMPONENTS
Enzyme Solution, 20S Proteasome	Store at -80 °C.* Avoid multiple freeze-thaw cycles.	Immediately place on ice and store for up to 24 hours. Once activated with SDS, activity is stable at 37°C for at least 1 hour.
20X Reaction Buffer	Store at -20°C.*	Store at room temperature.
100X Activation Solution		
1000X Substrate Solution		

* Provided this is within the expiration date of the kit.

OTHER MATERIALS REQUIRED

- Micro-centrifuge
- Fluorescence spectrophotometer
- Quartz cuvettes or flat-bottom opaque 96-well plate
- Free AMC for standard curve generation and sample quantitation; optional

CURVETTE ASSAY PROTOCOL

Kit contains enough reagents for 25 x 1 mL assays

1. Thaw all solutions briefly in a warm water bath. Make an appropriate amount of 1X reaction buffer for the number of experiments planned and place the reaction buffer at 37 °C.
2. Pipette 990 µL 1X reaction buffer into an eppendorf tube, add 10 µL 3% SDS solution and mix thoroughly.
3. Pipette 0.7 µL (~1 µg) 20S proteasome into the 0.03% SDS solution tube, mix, and transfer the enzyme solution to the cuvette. Allow 5-10 minutes for temperature equilibration.
4. Pipette 1 µL 1000X substrate solution into the cuvette, mix, and monitor fluorescence signal over time using excitation and emission wavelengths of 345 nm and 445 nm, respectively.

PLATE ASSAY PROTOCOL

Kit contains enough reagents for 1 x 96-well microtiter plate

1. Thaw all solutions briefly in a warm water bath. Using a 15 or 50 mL conical tube, prepare the amount of 1X Reaction Buffer needed for the number of reactions planned. Each well will require a total of 200 uL.
2. Add SDS to the reaction buffer such that final concentration is 0.03%.
3. Add proteasome enzyme (0.2 µg per well) and incubate for 5-10 minutes for equilibration at assaying temperature.
4. Using a multi-channel pipette, aliquot 190 µl activated enzyme mixture into each well on the plate and allow for temperature equilibration.
5. Make a 20X (200 µM) substrate stock solution [1:50] diluted in reaction buffer. Each well will require 10 µL.
6. Initiate the reaction by adding 10 µL 20X substrate solution to each well.
7. Monitor fluorescence signal over time using excitation and emission wavelengths of 345 nm and 445 nm, respectively.

SAMPLE DATA

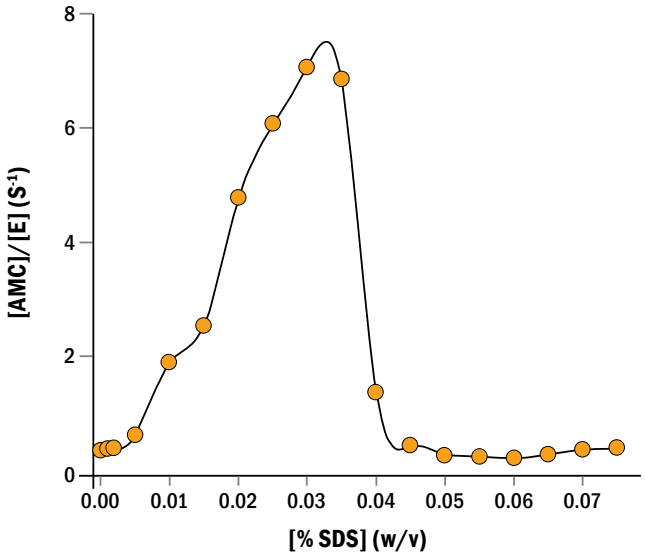

Figure 2: SDS-activation of purified 20S Proteasome. Proteasome (1.2 nM) was pre-incubated with reaction buffer (25 mM Hepes pH 7.6, 0.5 mM EDTA) with varying concentrations of SDS at 37oC. Substrate (20 µM Suc-LLYY-AMC) was added and hydrolysis (rate of AMC release) was monitored using excitation and emission wavelengths of 345 nm and 445 nm, respectively.

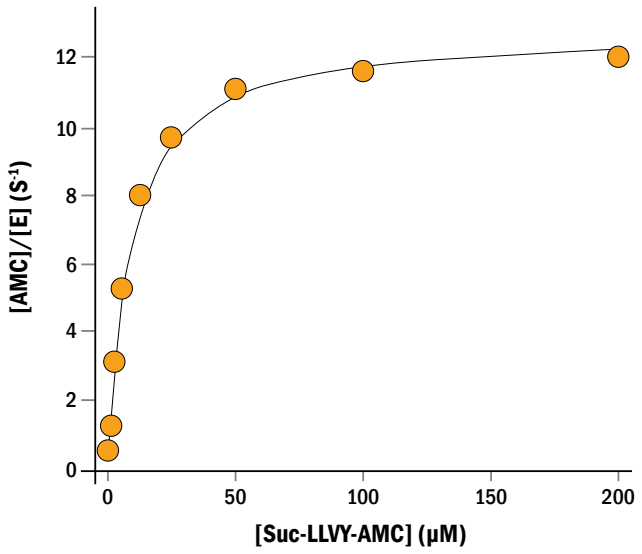

Figure 2: Michealis-Menton parameters of SDS-activated 20S Proteasome using LLVY-AMC Substrate. Proteasome (1.2 nM) was activated with reaction buffer (25 mM Hepes pH 7.6, 0.5 mM EDTA, 0.025% SDS) and assayed with varying concentrations of substrate (0.05-200 µM) at 37 °C. The rate of AMC release was monitored using excitation and emission wavelengths of 345 nm and 445 nm, respectively.

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