Proteasome Activity Fluorometric Assay Kit I
(Cat. # J4110)

* The supplied Suc-LLVY-AMC and 20S proteasome are sufficient for 165 X and 10 X 150 µl reactions, respectively.

**Description**

Proteasome Activity Fluorometric Assay Kit I is designed for assaying the proteasome’s chymotrypsin-like activity in vitro using purified proteasome, cleared cell lysates or tissue extracts. The proteasome cleaves SUC-LLVY-AMC and the released AMC fluorescence can be monitored using a plate reader or fluorometer at the excitation/emission wavelength of 360nm/460nm, respectively. The amount of the cell lysates or tissue extracts to be used in the reaction should be optimized.

AMC is used to produce a standard curve using a concentration range of 10-100 pmol.

Dissolve 1 mg MG132 in 105 µl DMSO to make a 20 mM stock solution before use, using 50-200 µM MG132 to inhibit the proteasome in vitro.

The bovine 20S proteasome can be used as a positive control. A typical 150 µl reaction contains 10 nM 20S proteasome, 50 µM fluorogenic substrate in the Proteasome Assay Buffer. The supplied latent 20S proteasome has low peptidase activities because the gate is closed. You can add 0.01-0.02% SDS into the reaction mixture to significantly stimulate its peptidase activities.

**Components**

- AMC (0.1mM in DMSO) 30 µl
- 1000X SUC-LLVY-AMC (50 mM in DMSO) 25 µl
- 40X Bovine 20S Proteasome (400 nM) 40 µl
- MG-132 1 mg
- DMSO 200 µl
- 20X Proteasome Assay Buffer 1.35 ml

20X Proteasome Assay Buffer: 800 mM Tris, pH 7.1 at 37°C, 40 mM βME

**Protocol for assaying proteasome activity using whole cell extracts**
The following protocol has been used to test the Proteasome Activity Fluorometric Assay Kit I and II using whole cell extracts from HeLa cells. You may optimize conditions for your specific assays.
[Prepare cell extracts]

1. One dish (100 mm) of HeLa cells was grown in DMEM supplemented with 10% fetal bovine serum to approximately 95% confluence. Cells were harvested and kept in a 15ml conical tube. Cells can be frozen in -80 °C freezer for future use.

2. Resuspend the cell pellet in 1 ml ice-cold cell lysis buffer (40 mM Tris, pH 7.2, 50 mM NaCl, 2 mM βME, 2 mM ATP, 5 mM MgCl₂, 10% Glycerol). Briefly sonicate cells using a 550 Sonic Dismembrator (Fisher Scientific). Settings: power output: 2, 20 seconds/time for three times, put the conic tube on ice for 2 min between sonications. Cells can also be lysed by using a homogenizer.

   Note: we did not add any protease inhibitors in the cell lysis buffer.

3. Ultracentrifuge the cell lysates using a 70.1Ti rotor (Beckman) at 36,000 rpm (100,000 Xg) for 30 min.

   Note: you may also centrifuge at 16,000 Xg for 20 min under 4 °C.

4. Carefully transfer the supernatant (avoid the lipids on the top) to a new 1.7 ml centrifuge tube and keep the supernatant on ice. We use a 10 ml syringe with a 21 gauge needle to poke through the lipid layer and take the supernatant. We recovered 0.8 ml supernatant.

5. Measure the supernatant concentration using the Bradford assay. We obtained a total of 3.4 mg cell extracts (4.3 mg/ml).

   Note: Fewer cells can be used to prepare the cell extracts by using less cell lysis buffer. We recommend a cell extract concentration to be around 5 mg/ml.

[Proteosomal peptidase assay]

6. Prepare 1.5 ml Suc-LLVY-AMC working solution. Mix 75 µl supplied Proteasome Assay Buffer (20X) with 1425 µl miliQ H₂O. Warm up in a 37 °C water bath for 10 min. Add 1.5 µL Suc-LLVY-AMC stock (1000 X) into the warmed buffer, vortex briefly to dissolve the substrate. Keep the substrate in a 37 °C water bath.

7. Set up a plate reader. We use a BioTek Synergy II plate reader with exciting and emission filters at 360/40 and 460/30 nm, respectively. For a kinetic assay, we use the following parameters: 15 min total reading time, 1 min/reading, sensitivity setting 60 and probing from the bottom of the plate. Warm up the instrument to 37 °C if your instrument has a temperature control function.

   Note: Each brand of plate reader is different, reading parameters may change accordingly. The following assay may help you to find an appropriate reading sensitivity and the amount of cell extracts to be used.

8. In a 96-well plate (we use black plates with flat and clear bottom from Corning, product# 3631), add 150 µl warmed substrate into each well for a total of 8 wells. In the four control wells, add 0, 10, 20 or 40 ul cell lysis buffer. In the four reaction wells, add 0, 10, 20, and 40 ul cell extracts obtained in step 5. Start plate reading immediately.
Note: you may adjust your plate reader sensitivity to obtain appropriate readings, which should give you a nice linear curve in samples with cell extracts and acceptable background readings in the control wells. Too high of sensitivity setting can increase background noise. In this case, you can reduce the sensitivity settings and increase your cell extract concentrations or volumes in the reactions to obtain appropriate readings. Too high concentrations of proteasome in the cell extracts may consume up the substrate rapidly. In this case, you can reduce the volume of cell extracts added in the assay or dilute your cell extracts.

9. After figure out plate reader sensitivity and the amount of cell extracts to be used, you can set up to read multiple samples. You can use the slope of the linear curves to reflect relative proteasome activities in different samples. Alternatively, you can use the supplied AMC to plot a concentration-dependent AMC fluorescence standard curve, then use the standard curve to calculate the absolute amounts of released AMC/min in your assays.