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Proteasome Activity Fluorometric Assay Kit I

(Cat. # J4110)

The supplied Suc-LLVY-AMC is sufficient for use in 250 X 100 µl reactions to monitor the chymotrypsin-like activity of constitutive proteasomes.

Description

Proteasome Activity Fluorometric Assay Kit I was designed for assaying constitutive proteasomes' chymotrypsin-like activity *in vitro* using purified proteasomes, cell lysates or tissue extracts. The proteasomes cleave Suc-LLVY-AMC, and the released AMC fluorescence can be monitored by using a plate reader or fluorometer at the excitation/emission wavelength of 360nm/460nm, respectively.

AMC can be used to generate a standard curve using a concentration range of 0 - 200 pmol, which can then be used to quantitate proteasome activities.

We recommend using the proteasome inhibitor MG132-treated samples as the blanks. In addition to preferred proteasomes, other enzymes in cell/tissue extracts may also cleave them at a slow rate. So MG132-treated cell/tissue extracts can be used as the blanks to deduct enzyme activities contributed by non-proteasome cleavage.

MG132 preparation: dissolving the supplied 1 mg MG132 in 105 µl DMSO to make a 20 mM stock solution. Using 100 µM final MG132 concentration to inhibit proteasomes *in vitro*.

HEK293T cell lysates can be used as a positive control. A typical 100 µl reaction contains 10 µl supplied HEK293T cell lysates and 50 µM fluorogenic substrate in the 1X Proteasome Assay Buffer (supplied as 20X stock). Aliquot the cell lysates to 10 µl to avoid freeze/thaw cycles.

Components

• AMC (0.1 mM in DMSO)	30 µl
• 1000X SUC-LLVY-AMC (50 mM in DMSO)	25 µl
• HEK293T cell lysates (5 mg/ml)	100 µl
• MG132	1 mg
• DMSO	200 µl
• ATP (500 mM)	100 µl
• $MgCl_2$ (1 M)	250 µl
• 20X Proteasome Assay Buffer	1.35 ml

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

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A protocol for assaying the proteasome activity using whole cell lysates

The following protocol was used to test the Proteasome Activity Fluorometric Assay Kit I. *Please optimize assay conditions for your experiments*.

[Prepare cell extracts]

1. Two dishes (100 mm) of HEK293T cells were grown in DMEM supplemented with 10% fetal bovine serum to approximately 95% confluence. Cells were harvested, washed twice with cold 1X PBS, and kept in a 15 ml conical tube. Cells can be frozen in a -80 °C freezer for future use.

2. Resuspend the cell pellet in 2 ml ice-cold cell lysis buffer (40 mM Tris, pH 7.2, 50 mM NaCl, 2 mM βME, 2 mM ATP, 5 mM $MgCl_2$, 10% glycerol). Briefly sonicate cells using a 550 Sonic Dismembrator (Fisher Scientific). Settings: power output: 3, 15 seconds/time for three times, cool on ice for 2 min after each sonication.
   Note: we did not add any protease inhibitors in the cell lysates because they may inhibit proteasomes. The 26S proteasomes are large protein complexes that can be dissociated or damaged by harsh buffer conditions, including the presence of detergents, high concentrations of salt, or heating (> 37 ^{0}C). So, avoid these reagents and conditions. Also, including 2 mM ATP and 5 mM $MgCl_2$ (provided as stocks) in the lysis buffer can preserve the 26S proteasome complexes.

3. Centrifuge the cell lysates using a refrigerated desktop centrifuge at 17,000 x *g* for 20 min under 4 ^{0}C.
   Note: you may also ultracentrifuge the cell lysates at 100,000 x *g* for 30 min under 4 ^{0}C.

4. Transfer the supernatant to a new 2 ml centrifuge tube and keep on ice. Measure the supernatant concentration using the Bradford assay. We obtained 4.6 mg/ml whole cell lysates.
   Note: We recommend a cell lysate concentration at 2-5 mg/ml for proteasome activity assays.

[Monitor proteasome activity using a plate reader]

5. Prepare 2X Suc-LLVY-AMC in Assay Buffer. Mix 50 µl supplied Proteasome Assay Buffer (20X) with 950 µl milliQ water. Warm up in a 37 ^{0}C water bath for 10 min. Add 2 µL Suc-LLVY-AMC stock (1000 X) into the warmed buffer, vortex 10 seconds to dissolve the substrate. At this step, the prepared substrate concentration is 100 µM (2X). Keep the substrate in a 37 ^{0}C water bath.
   Note: Prepare appropriate amounts of substrate according to how many assays you plan to do. 50 µl 2X substrate will be used in each assay.

6. Set up a plate reader. We use a BioTek Synergy II plate reader with excitation and emission filters at 360/40 and 460/30 nm, respectively. We use the following parameters in a 15 min kinetic assay: 1 min per reading interval, sensitivity setting at 62, probing from the clear bottom of the plate, and shaking the plate for 5 seconds before the first reading to mix samples. Warm up the instrument and the 96-well plate to 37 ^{0}C.
   Note: Each brand of plate reader is different, reading parameters may change accordingly. The following assay in **Step 7** can find an appropriate reading sensitivity and the amount of cell lysates to be used.

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7. Determine the instrument sensitivity setting and the amount of cell lysates to be used. We use a 96-well black plate with flat and clear bottom manufactured by Corning (catalog# 3631). Add 50 μl warmed substrate prepared in **Step 5** into each well for a total of 2 wells. In the first well, add an additional 50 μl 1X Assay Buffer (supplied as 20X stock). In the second well, add 50 μl cell lysates (we premixed 10 μl cell lysates prepared in **Steps 1-4** and 40 μl 1X Assay Buffer, and kept in a 37 ^{0}C water bath for 10 min). Recording AMC fluorescence immediately.
   Note: You may adjust your plate reader sensitivity to obtain appropriate readings, which should show a nice linear curve in the well with cell lysates and acceptable substrate background readings in the control well. Too high of sensitivity setting can increase substrate background reading. In this case, you can reduce the sensitivity setting. *Vice versa*, you can increase the sensitivity setting if the fluorescent readings are too low. Also, too high concentration of proteasomes in the cell lysates may consume up the substrate rapidly (you observe a rapid increase of AMC fluorescence in the first a few minutes, and then the fluorescence signal reaches a plateau or over the detection limit of your instrument). In this case, you can reduce the amount of cell lysates or reduce the detection sensitivity. Once an assay condition is changed, repeat the assay to determine if the change is appropriate.

8. Prepare MG132-treated and non-treated cell lysates. We added 1.25 μl MG132 stock (20 mM in DMSO) into warmed 200 μl 1X Assay Buffer in a 1.5 ml centrifuge tube, vortex to mix, and then added 50 μl cell lysates prepared in **Steps 1-4**. The final MG132 concentration in the mixture was 100 μM. In another 1.5 ml centrifuge tube, we mixed 50 μl cell lysates with 200 μl 1X Assay Buffer. Keep both samples in a 37 ^{0}C water bath for 10 min.

9. In the meanwhile, you should set up your plate reader ready for the assay.

10. Assay peptidase activity. We performed triplicates for each condition. Add 50 μl MG132-treated cell lysates to the first 3 wells, and non-treated cell lysates to another 3 wells, then add 50 μl Suc-LLVY-AMC substrate prepared in **Step 5** into each well. Record AMC fluorescence immediately in a 15-min kinetic mode.
    Note: If you have multiple samples, you can add your various samples (MG132-treated or non-treated) in a 96-well plate first, then use a multi-channel pipette to add the substrate quickly.

    We do not recommend using an end point assay to monitor proteasome activity because you may not know if a reaction is saturation.

[Data Analyses]

11. We use the slope value of each curve to reflect the relative proteasome activity in each sample. Fig. 1 showed the actual reading curves in our assay. Wells 1-3 were MG132-treated cell lysates; wells 4-6 were non-treated cell lysates. We exported reading values in well 1 (MG132 treated) and well 4 (non-treated), and replotted (Fig. 2). In this specific case, the reading exceeded the detection limit of our instrument after 11 min in cell lysate-containing wells (Fig. 2).

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Fig. 1.

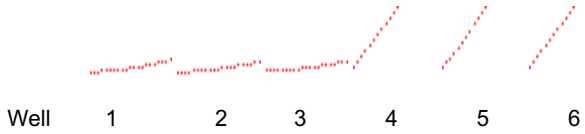
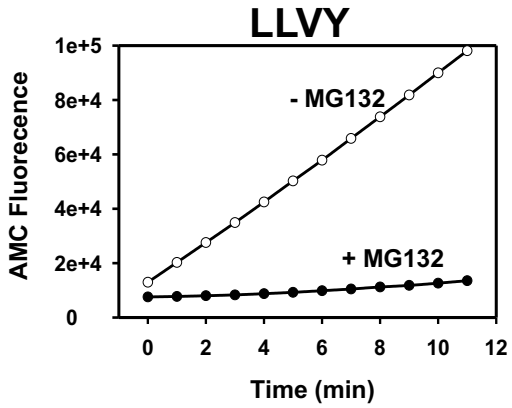


Fig. 2.



12. AMC fluorescence in either MG132-treated or non-treated assays were in linear growth in the 11 min assay window. We used the first 10 min reading curves to calculate the slope values. The slope value of each curve was calculated by using the formula $(Y_{10}-Y_0)/(X_{10}-X_0)$, in which Y_{10} and Y_0 were AMC fluorescence readings from Y axis at 10 min and 0 min, respectively. X10 - X0 = 10 min. In Fig. 2, the slope value of MG132-treated or non-treated one was 5.14 X 10^5 fluorescent units (FU)/min or 7.72 x 10^6 FU/min, respectively. The relative proteasome chymotrypsin-like activity of the 293T cell lysate was (7.72 - 0.514) X 10^6 FU/min = 7.21 x 10^6 FU/min. The other two reactions in Fig. 1 had similar proteasome activity at 7.13 x 10^6 FU/min and 7.24 x 10^6 FU/min, respectively.

    Using triplicates (three reactions for each sample and three samples for each of your experimental subject), you are able to obtain statistical analyses. This also allow you to compare proteasome activities in different samples. For example, comparing proteasome activities in wild type vs. mutant cell lines.

13. Alternatively, you can use the supplied AMC to generate a concentration-dependent AMC fluorescence standard curve. For example, using 0, 5, 10, 20, 40, 80 and 160 pmol AMC in 100 µl assay buffer to record AMC fluorescence at the same sensitivity setting, then plotting AMC fluorescence (y axis) vs. AMC concentration (x axis) to generate a standard curve. The standard curve can be used to calculate the absolute amounts of released AMC in each reaction. In this case, you need to choose reading values at a time point of the kinetic reading at which all sample kinetic curves are at linear growth.