

Proteasome Activity Fluorometric Assay Kit II

(Cat. # J4120)

Each supplied substrate is sufficient for use in 250 X 100 μ l reactions to monitor the chymotrypsin-like (Suc-LLVY-AMC), trypsin-like (Boc-LRR-AMC) or caspase-like (Z-LLE-AMC) activity of constitutive proteasomes.

Description

Proteasome Activity Fluorometric Assay Kit II was designed for assaying constitutive proteasomes' three peptidase activities *in vitro* using purified proteasomes, cell lysates or tissue extracts. The proteasomes cleave these fluorogenic substrates, and the released AMC fluorescence can be monitored 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 slow rates. 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 μ l DMSO to make a 20 mM stock solution. Using 100 μ M final MG132 concentration to inhibit proteasomes *in vitro*.

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

Components

- | | |
|--------------------------------------|-------------|
| • AMC (0.1 mM in DMSO) | 30 μ l |
| • 1000X Suc-LLVY-AMC (50 mM in DMSO) | 25 μ l |
| • 1000X Boc-LRR-AMC (50 mM in DMSO) | 25 μ l |
| • 1000X Z-LLE-AMC (50 mM in DMSO) | 25 μ l |
| • HEK293T cell lysates (5 mg/ml) | 100 μ l |
| • MG132 | 1 mg |
| • DMSO | 200 μ l |
| • ATP (500 mM) | 100 μ l |
| • MgCl ₂ (1 M) | 250 μ l |
| • 20X Proteasome Assay Buffer | 3 X 1.35 ml |

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



A protocol for assaying the proteasome activity using whole cell lysates

The following protocol was used to test the Proteasome Activity Fluorometric Assay Kit II. *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 β ME, 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, put the conic tube 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, and/or heating (> 37 °C). So, avoid these reagents or 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 °C.
Note: you may also ultracentrifuge the cell lysates at 100,000 x *g* for 30 min under 4 °C.
4. Transfer the supernatant to a new 2 ml centrifuge tube and keep on ice. Determine the supernatant concentration using the Bradford assay. We obtained 4.6 mg/ml whole cell lysates.
Note: We recommend a cell lysate concentration at ~5 mg/ml for proteasome activity assays.

[Monitor proteasome activity using a plate reader]

5. Prepare 2X substrates in Assay Buffer. Mix 50 μ l supplied Proteasome Assay Buffer (20X) with 950 μ l milliQ water. Warm up in a 37 °C water bath for 10 min. Add 2 μ l 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 μ M (2X). Keep the substrate in a 37 °C water bath. Similar approach was used to prepare 100 μ M Z-LLE-AMC and Boc-LRR-AMC in 1X Assay Buffer.

Note: Prepare appropriate amounts of substrates according to how many assays you plan to do. 50 μ l 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. The following parameters were used for a 15 min kinetic assay: 1 min per reading interval, sensitivity setting at 62, probing from the 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 °C.
Note: Each brand of plate reader is different, reading parameters may change accordingly. The following assay in **Step 7** may help to find an appropriate reading sensitivity and cell lysate amounts to be used.



- Determine the instrument sensitivity setting and the amount of cell lysates to be used in each assay. We use a 96-well black plate with flat and clear bottom manufactured by Corning (catalog# 3631). Add 50 μ l each warmed substrate prepared in **Step 5** into each well, two wells for each substrate. For each substrate, add an additional 50 μ l 1X Assay Buffer (supplied as 20X stock) to one well as background of the substrate itself. In the second well, add 50 μ l cell lysates (we premixed 10 μ l cell lysates prepared in **Steps 1-4** and 40 μ l 1X Assay Buffer, and kept in a 37 °C water bath for 10 min). This well was used to monitor proteasome activity. 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 the sensitivity setting can increase substrate background reading. In this case, you can reduce the sensitivity setting. *Vice versa*, you may need to increase the detection sensitivity if the reading values are too low. You will notice that Z-LLE-AMC and Boc-LRR-AMC have much higher background reading than Suc-LLVY-AMC. If you want to assay all three substrates in the same 96-well plate, you need to find an appropriate sensitivity setting that fits for monitoring activities based on all three substrates.

In addition, 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 the fluorescence signal then 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.

- Prepare MG132-treated and non-treated cell lysates. We added 1.25 μ l MG132 stock (20 mM in DMSO) into warmed 200 μ l 1X Assay Buffer in a 1.5 ml centrifuge tube, vortex to mix, and then added 50 μ l cell lysates prepared in **Steps 1-4**. The final MG132 concentration in the mixture was 100 μ M. In another 1.5 ml centrifuge tube, we mixed 50 μ l cell lysates with 200 μ l 1X Assay Buffer. Keep both samples in a 37 °C water bath for 10 min.
- In the meanwhile, set up your plate reader ready for the assay.
- Assay peptidase activity. We performed triplicates for each condition. Add 50 μ l MG132-treated cell lysates to the first 3 wells, and non-treated cell lysates to the other 3 wells, then add 50 μ l Suc-LLVY-AMC substrate prepared in **Step 5** into each well. Recording AMC fluorescence immediately in a kinetic mode (15 min reading). Similar assays were done for Z-LLE-AMC or Boc-LRR-AMC. We monitored proteasome activities of all three substrates in one 96-well plate.

Note: If you have multiple samples, you can add your samples (MG132-treated or non-treated) in a 96-well plate first. Arranging reactions with the same substrate in the same row or column of a 96-well plate would allow you to quickly add the substrate using a multi-channel pipette.

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

[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 with Suc-LLVY-AMC as the substrate. 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 lysates wells (Fig. 2). Similar curves were replotted with Z-LLE-AMC or Boc-LRR-AMC being the substrate (Fig. 2)

Fig. 1.

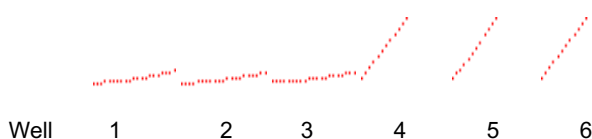
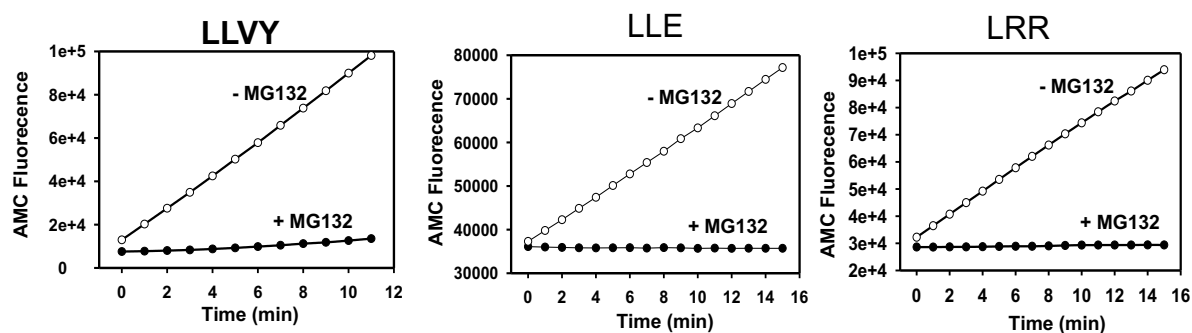


Fig. 2.



12. AMC fluorescence in either MG132-treated or non-treated assays were in linear growth in the kinetic 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. $X_{10} - X_0 = 10$ min. In Fig. 2, the slope values of MG132-treated and non-treated one were 5.14×10^5 fluorescent units (FU)/min and 7.72×10^6 FU/min, respectively. So the relative proteasome chymotrypsin-like activity of the 293T cell lysate was $(7.72 - 0.514) \times 10^6$ FU/min = 7.21×10^6 FU/min. The other two reactions in Fig. 1 had similar final proteasome activity at 7.13×10^6 FU/min and 7.24×10^6 FU/min, respectively, for reactions with Suc-LLVY-AMC as the substrate.

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 μ l 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 sample. 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.

