Immunoproteasome Activity Fluorometric Assay Kit II (Cat. # J4170)

Each supplied substrate is sufficient for use in 250 X 100 µl reactions to monitor the chymotrypsin-like (Ac-ANW-AMC), trypsin-like (Ac-KQL-AMC), or branched amino acid preferring (Ac-PAL-AMC) activity of immunoproteasomes. Ac-ANW-AMC and Ac-PAL-AMC are preferred substrates of immunoproteasomes compared to constitutive proteasomes. Ac-KQL-AMC can be efficiently cleaved by both immunoproteasomes and constitutive proteasomes.

Description

Immunoproteasome Activity Fluorometric Assay Kit II was designed for assaying immunoproteasomes' chymotrypsin-like, trypsin-like and branched amino acid preferring activities *in vitro* using purified proteasomes, cell lysates or tissue extracts. The immunoproteasomes cleave these substrates, 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 the substrates. 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*.

Mouse spleen extracts (containing > 90% immunoproteasomes) can be used as a positive control. A typical 100 µl reaction contains 10 µl supplied mouse spleen extracts and 50 µM fluorogenic substrate in the 1X Proteasome Assay Buffer (supplied as 20X stock). Aliquot the extracts to 10 µl to avoid freeze/thaw cycles.

Components

- AMC (0.1 mM in DMSO)   30 µl
- 1000X Ac-ANW-AMC (50 mM in DMSO)   25 µl
- 1000X Ac-PAL-AMC (50 mM in DMSO)   25 µl
- 1000X Ac-KQL-AMC (50 mM in DMSO)   25 µl
- Mouse spleen extract (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   3 X 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 immunoproteasome activity using mouse spleen extracts

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

[Prepare tissue extracts]

1. Two mouse spleens (0.2 g total) were harvested and snap frozen in liquid nitrogen. Cool a mortar and pestle using liquid nitrogen. Transfer spleens into a mortar with liquid nitrogen, grind thoroughly until observing dry powders (can continuously grind with liquid nitrogen). Add small amount of liquid nitrogen, and transfer powders into a 15 ml conical tube.

2. Resuspend the spleen powders 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 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 protease inhibitors in the extracts as they may inhibit proteasome activities. 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 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 extracts using a refrigerated desktop centrifuge at 17,000 x *g* for 20 min under 4 ^{0}C.
   Note: you may also ultracentrifuge the extracts at 100,000 x *g* for 30 min under 4 ^{0}C.

4. Transfer the supernatant into a new 2 ml centrifuge tube and keep on ice. Measure the supernatant concentration using the Bradford assay. We obtained 6.6 mg/ml whole spleen extracts.
   Note: We recommend an extract concentration at ~5 mg/ml for proteasome activity assays.

[Monitor proteasome activity using a plate reader]

5. Prepare 2X fluorogenic substrate 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 Ac-ANW-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. The same approach was used to prepare 100 μM Ac-KQL-AMC or Ac-PAL-AMC.
   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 60, 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.

7. 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 µl each warmed substrate prepared in **Step 5** into each well, two wells for each substrate. For each substrate, add an additional 50 µl 1X Assay Buffer (supplied as 20X stock) to one well as background of the substrate itself. 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). 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 Ac-PAL-AMC and Ac-KQL-AMC have much higher background reading than Ac-ANW-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.

8. Prepare MG132-treated and non-treated extracts. 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 extracts 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 extracts with 200 µl 1X Assay Buffer. Keep both samples in a 37 ^{0}C water bath for 10 min.

9. In the meanwhile, 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 the other 3 wells, then add 50 µl Ac-ANW-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 Ac-KQL-AMC or Ac-PAL-AMC. We monitored proteasome activities of all three substrates together 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. Wells 1-3 were MG132-treated extracts; wells 4-6 were non-treated extracts. We exported reading values in well 1 (MG132 treated) and well 4 (non-treated), and replotted (Fig. 2). The same approach was used for Ac-KQL-AMC or Ac-PAL-AMC-based assays (Fig. 2)
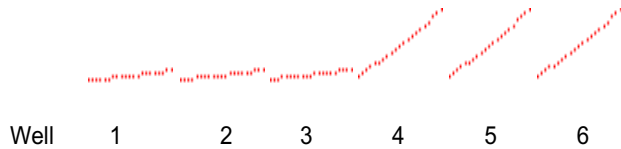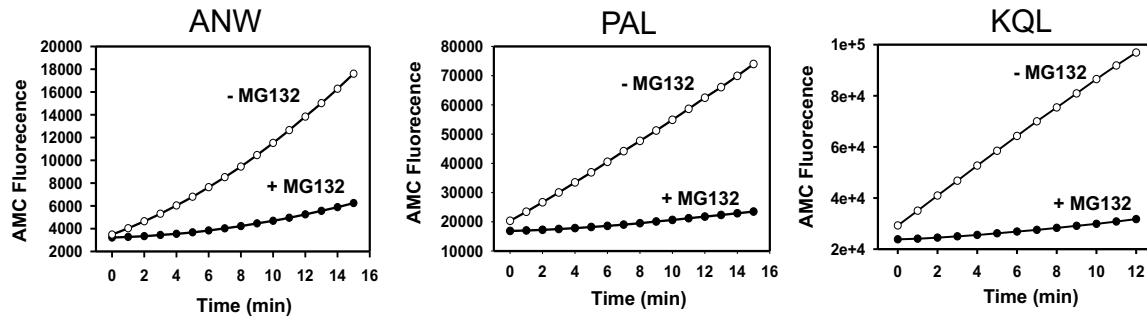
Fig. 1.

Well   1   2   3   4   5   6

Fig. 2.

12. AMC fluorescence in either MG132-treated or non-treated assays were in linear growth in the 15-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. $X_{10} - X_0 = 10$ min. In Fig. 2, the slope value of MG132-treated or non-treated one was 1.48×10^5 fluorescent units (FU)/min or 8.2×10^5 FU/min, respectively. The relative proteasome chymotrypsin-like activity of the mouse spleen extracts was $(8.2-1.48) \times 10^5$ FU/min $= 6.72 \times 10^5$ FU/min. The other two reactions in Fig. 1 had similar proteasome activity at 6.54×10^5 FU/min and 6.62×10^5 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.