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Proteasome Activation (Assembly) Fluorometric Assay Kit

Cat. # J4000

The supplied fluorogenic substrate Mca-KKVAPYPME-Dap(Dnp)-NH_2 is sufficient for 500 X 50 µl reactions to monitor the activity of activated proteasomes from mammalian, yeast or archaea.

Description

The Proteasome Activation (Assembly) Fluorometric Assay Kit Can be used for assaying 20S proteasome activation (gate opening) by assembling with a regulatory particle or a chemical.

Mca-KKVAPYPME-Dap(Dnp)–NH_2 is a fluorogenic peptide substrate of proteasomes, in which the Mca fluorescence is internally quenched by Dap(Dnp). This substrate can be cleaved by mammalian, yeast or archaea activated proteasomes. Upon cleavage, the Mca fluorescence can be measured at excitation/emission wavelengths at 320/405 nm using a plate reader or fluorometer. Due to its long sequence as a nanomer, latent 20S proteasome has a weak activity on hydrolysis of Mca-KKVAPYPME-Dap(Dnp)–NH_2, thereby it is a better substrate than Tri or tetra-peptide substrates for monitoring gate opening of 20S proteasomes.

To avoid photobleaching in kinetic assays, end point assays can be used once you have optimized assay conditions to ensure the Mca fluorescence is under linear increase at the end of the time point in your end point assay.

Kit Components

• 1000X Mca-KKVAPYPME-Dap(Dnp)-NH_2 (20 mM in DMSO)	25 µl
• MG132	1 mg
• DMSO	200 µl
• ATP (500 mM)	100 µl
• $MgCl_2$ (1 M)	250 µl
• NaCl (1M)	500 µl
• 20X Proteasome Assay Buffer	1.35 ml

20 mM MG132 stock preparation: dissolve the supplied 1 mg MG132 in 105 µl DMSO to make a 20 mM stock solution. Use 100 µM final MG132 concentration to inhibit proteasomes *in vitro*. MG132-treated samples can be used to inhibit proteasome activity if necessary.

20X Proteasome Assay Buffer: 800 mM Tris, pH 7.1 at 37°C (pH 7.6 at 4 ^{0}C), 40 mM βME

A protocol for assaying activation of the 20S proteasome by the 19S regulatory particle (PA700)

Please optimize assay conditions for your specific experiments.

1. Prepare 500 µL Proteasome Assembly Buffer: 40 mM Tris, pH 7.6 at 4 ^{0}C, 20 mM NaCl, 2 mM βME, 2 mM ATP, 5 mM $MgCl_2$, 10% glycerol.
   Note: You can use the supplied 20S Proteasome Assay Buffer to make the Proteasome Assembly Buffer. Glycerol not included.

2. Prepare 100 μL 8 nM bovine 20S proteasome (UBPBio catalog # A1400) or 8 nM bovine 26S proteasome (UBPBio catalog # A1200) in cold Proteasome Assembly Buffer in a 1.5 ml centrifuge tube. Also prepare 100 μL 8 nM bovine 20S proteasome + 64 nM 19S regulatory particle (UBPBio catalog # A1300) in cold Proteasome Assembly Buffer in another 1.5 ml centrifuge tube. Gently tap the tubes to mix well, then incubate in a 37 ^{0}C water bath for 30 min.

   Note: we recommend add 5-10-fold (concentration vs. concentration) more regulatory particles than the 20S proteasome to stimulate assembly of the proteasome complexes.

3. Prepare 2X Mca-KKVAPYPME-Dap(Dnp)-NH_2 in Assay Buffer. Mix 20 μl supplied Proteasome Assay Buffer (20X) with 380 μl milliQ water. Warm up in a 37 ^{0}C water bath for 10 min. Add 0.8 μL Mca-KKVAPYPME-Dap(Dnp)-NH_2 stock (20 MM in DMSO) into the warmed buffer, vortex vigorously for 20 seconds to dissolve the substrate. At this step, the prepared substrate concentration is 40 μM (2X). Keep the substrate in a 37 ^{0}C water bath.

4. Set up a plate reader. We use a BioTek H2 plate reader with the excitation and emission wavelengths at 320 and 405 nm, respectively. We use the following parameters in a 30 min kinetic assay: 1 min per reading interval, sensitivity setting at 65, probing from the clear bottom of the plate, and shaking the plate for 8 seconds before the first reading to mix samples. Warm up the instrument and the 96-well plate at 37 ^{0}C.

   Note: Each brand of plate reader is different, reading parameters may change accordingly.

5. Assay peptidase activity. We use a 96-well black plate with flat and clear bottom manufactured by Corning (catalog# 3631).The following conditions were tested: 1) add 25 μl 2X Proteasome Assembly Buffer prepared in **Step 1** into each well (three wells total); 2) add 25 μl 20S proteasome prepared in **Step 2** into each well (three wells total); and 3) add 25 μl 20S proteasome + 19S regulatory particle prepared in **Step 2** into each well (three wells total). Add 25 μl 2X Mca-KKVAPYPME-Dap(Dnp)-NH_2 substrate prepared in **Step 3** into each well. Record the Mca fluorescence immediately in a 30-min kinetic mode.

   Note: If you have multiple samples, you can add samples into a 96-well plate first, then use a multi-channel pipette to add the substrate quickly.

6. Data were plotted with X axis as time vs. Y axis as the Mca fluorescence (A). The slope value of each curve was calculated and used to represent activity of each sample. Data were mean ± S.D. (B).