Proteasome Activity Fluorometric Assay Kit

(Catalog #K245-100; 100 assays; Store kit at -20°C)

I. Introduction:

Proteasomes are very large (20S, 26S) protein assemblies found in both the nucleus and cytoplasm of all eukaryotes (and in some prokaryotes). They are responsible for the degradation and recycling of proteins which have been previously tagged with ubiquitin. Such tagged proteins are degraded into peptides approximately 7-8 amino acids long which are subsequently further degraded. The 20S assembly is the functional protease structure with chymotrypsin-like, trypsin-like and caspase-like protease activities. **BioVision's Proteasome Activity Assay** takes advantage of the chymotrypsin-like activity, utilizing an AMC-tagged peptide substrate which releases free, highly fluorescent AMC in the presence of proteolytic activity. The kit also includes a positive control (Jurkat Cell lysate with significant proteasome activity) and a specific proteasome inhibitor MG-132 which suppresses all proteolytic activity due to proteasomes. This permits differentiation of proteasome activity from other protease activity which may be present in samples.

II. Kit Contents:

Components	100 assays	Cap Code	Part Number
Proteasome Assay Buffer	25 ml	WM	K245-100-1
Proteasome Substrate (Succ-LLVY-AMC in DMSO)	100 µl	Red	K245-100-2
Proteasome Inhibitor (MG-132 in DMSO)	100 µl	Blue	K245-100-3
AMC Standard (1 mM in DMSO)	100 µl	Yellow	K245-100-4
Positive Control	lyophilized	Green	K245-100-5

III. Storage and Handling:

Store the kit at -20°C, protect from light. Read the entire protocol before performing assay. Avoid repeated freeze/thaw cycles. All Samples and the Positive Control should be assayed in duplicate, (once in the absence and once in the presence of the Proteasome Inhibitor). An opaque, white, microwell plate is recommended. This protocol is designed for use in a 96 well plate. 384-well plates may be used but all reagent amounts should be reduced 5-fold (diluted if necessary). ***Do not use protease inhibitors during cell lysate preparation.***

Proteasome Substrate, Proteasome Inhibitor, AMC Standard: Ready to use as supplied. These DMSO solutions must be warmed to room temperature (RT) prior to use to melt frozen DMSO. We recommend warming in a 37°C water bath, pipetting up and down to ensure they are completely melted and mixed before use. Store at -20°C in the dark when not in use.

Positive Control: Reconstitute with 100 µl dH_2O. If kit will be used multiple times over an extended period of time, aliquot portions and store at -80°C. Keep on ice while in use. Avoid repeated freeze/thaw cycles.

IV. Assay Protocol:

1. **AMC Standard Preparation:** Dilute AMC Standard 100-fold (10 µl + 990 µl dH_2O) then add 0, 2, 4, 6, 8, 10 µl of AMC standard to a series of microplate wells. Adjust volume to 100 µl/well with Assay Buffer to generate 0, 20, 40, 60, 80 and 100 pmol per well AMC Standard.
2. **Positive Control Preparation:** Add 10 µl of the Positive Control to paired wells. Bring volume to total 100 µl by adding 90 µl of Assay Buffer to each well.
3. **Samples:** Prepare by homogenizing cells with 0.5 % NP-40 in dH_2O or PBS. Add up to 50 µl of each cell extract or other proteasome sample to be tested to paired wells. Bring the volume of each well to 100 µl with Assay Buffer. **Note:** For Unknown Samples, we suggest doing pilot experiment and testing several doses to ensure the readings are within the Standard Curve range.
4. **Inhibitor:** Add 1 µl of the Proteasome inhibitor to one of the paired wells, 1 µl of Assay Buffer to the other well, mix.
5. **Substrate:** Add 1 µl of Proteasome Substrate to all the Sample and Positive Control wells. Mix, protected from light.
6. **Read:** Measure kinetics of fluorescence development at Ex/Em = 350/440 nm in a microplate reader at 37°C for 30-60 min. There is a slight lag and nonlinearity to the kinetics due to the time it takes for the reaction mix to warm up to 37°C. Measurement of the wells which do not contain Proteasome Inhibitor will show total proteolytic activity RFU_1 and the wells containing Proteasome Inhibitor will show non-proteasome activity $iRFU_1$ at T_1. Measure RFU_2 and $iRFU_2$ at T_2 after 30 min (or longer time if the sample activity is low). The RFU generated by proteasome activity is $\Delta RFU = (RFU_2 - iRFU_2) - (RFU_1 - iRFU_1)$.

   Note: It is essential to read RFU_1, $iRFU_1$, RFU_2 and $iRFU_2$ in the linear reaction range. It will be more accurate if you monitor the reaction kinetics as shown in Fig. 1B. Then choose T_1 and T_2 in the appropriate linear range. From our experience, initial readings RFU_1 and $iRFU_1$ should be measured after ~ 20-25 min.
7. **Calculation:** Plot the AMC Standard Curve. Apply the ΔRFU to the AMC Standard Curve to get B pmol of AMC (amount generated between T_1 and T_2 in the reaction wells specifically by proteasome activity).

Proteasome Activity = $\frac{B}{(T2-T1)\times V}$ **× Sample Dilution Factor = nmol/min/ml = U/ml**

Where: **B** is the AMC amount from AMC Standard Curve (in pmol)
T_1 is the time of the first reading (RFU_1 and $iRFU_1$) (in min)
T_2 is the time of the second reading (RFU_2 and $iRFU_2$) (in min)
V is the pretreated Sample volume added into the reaction well (in µl)

Proteasome Unit Definition: One unit of proteasome activity is defined as the amount of proteasome which generates 1.0 nmol of AMC per minute at 37°C.

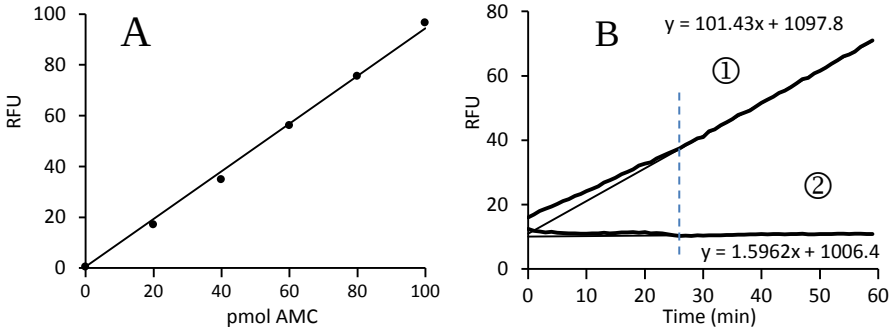

Fig. 1. AMC Standard Curve and Proteasome Activity assay using the kit protocol:
A: AMC Standard Curve 0-100 pmole; **B:** Kinetics of Proteasome Activity assay in the absence ① and presence ② of MG-132 Proteasome inhibitor. Equations represent best fit of lines during the linear portion of the reaction (after ~ 25 min in this case).

RELATED PRODUCTS:

Jurkat Cell Extract	Caspase, Cathepsin, Calpain active proteins
Caspase and Cathepsin Inhibitors	Proteasome/Calpain Substrate and Assay Kits
Caspases: Substrates and Assay Kits	MG-132 Proteasome Inhibitor
Cathepsins: Substrates and Assay Kits	Protease Inhibitor Cocktails
EZBlock™ Protease Inhibitor Cocktails	Cell Fractionation Kits

FOR RESEARCH USE ONLY! Not to be used on humans.

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GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer • Omission of a step in the protocol • Use of a different 96-well plate	• Assay buffer must be at room temperature • Refer and follow the data sheet precisely • Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type • Samples prepared in a different buffer • Cell/ tissue samples were not completely homogenized • Samples used after multiple free-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples	• Refer data sheet for details about incompatible samples • Use the assay buffer provided in the kit or refer data sheet for instructions • Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope • Aliquot and freeze samples if needed to use multiple times • Troubleshoot if needed, deproteinize samples • Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used	• Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer datasheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components • Pipetting errors • T1 readings too early • Air bubbles formed in well • Standard stock is at an incorrect concentration • Calculation errors • Substituting reagents from older kits/ lots	• Thaw and resuspend all components • Avoid pipetting small volumes • Usuually wait ~ 25 min before reading T1 (to get past lag phase) • Pipette gently against the wall of the tubes • Always refer the dilutions in the data sheet • Recheck calculations after referring the data sheet • Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength • Samples contain interfering substances • Use of incompatible sample type • Sample readings above/below the linear range	• Check the equipment and the filter setting • Troubleshoot if it interferes with the kit • Refer data sheet to check if sample is compatible with the kit or optimization is needed • Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		

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