

ASSAY PROTOCOL

20S Proteasome Fluorimetric Substrate Assay

Assay Buffer

Prepare 100mL of buffer with the following composition:

50mM Tris pH 7.5

25mM KCl

10mM NaCl

1mM MgCl₂

Substrate solutions

Prepare a 10mM stock solution of each substrate by dissolving (MW/1000)mg in 100µL DMSO.

Suc-LLVY-AMC	BIOMOL P802	MW 763	763µg/mL DMSO
Bz-VGR-AMC	BIOMOL BW9375	MW 706	706µg/mL DMSO
Z-LLE-AMC	BIOMOL ZW9345	MW 665	665µg/mL DMSO

For **Suc-LLVY-AMC** substrate:

Add 20µL of the stock solution drop-wise to 980µL of rapidly stirring (vortexing) **Assay Buffer** to give a clear solution at 200µM.

For **Bz-VGR-AMC** and **Z-LLE-AMC** substrates:

Add 40µL of either stock solution drop-wise to 960µL of rapidly stirring (vortexing) **Assay Buffer** to give a clear solution at 400µM.

AMC/substrate standards

Please note that due to auto-hydrolysis of AMC-containing peptide substrates it is necessary to carry out standard curves in the presence of substrates.

Dissolve AMC (17.5mg) in DMSO (1.0mL) to give a 100mM solution [**AMC Solution**].

Dissolve substrates in DMSO (1.0mL) to prepare 3mM substrate solutions.

Suc-LLVY-AMC 2.29mg [**Substrate S Solution**]

Bz-VGR-AMC 2.12mg [**Substrate B Solution**]

Z-LLE-AMC 2.0mg [**Substrate Z Solution**]

Add **AMC solution** (5µL) to 1mL of each of the three substrate solutions to prepare **Substrate-S-AMC**, **Substrate-B-AMC** and **Substrate-Z-AMC** solutions, respectively.

Add 1mL of *either* **Substrate-S-AMC**, **Substrate-B-AMC** or **Substrate-Z-AMC** DMSO solutions drop-wise to the centre of vortexing **Assay Buffer** (29.0mL) to give a clear solution at 16.7µM AMC and 100µM substrate (ensure complete transfer of AMC/substrate DMSO solutions). Repeat with the remaining substrate-AMC solutions until you have 3 bottles each containing 30mL of **AMC/Substrate** solutions in **Assay Buffer**.

The solutions should be used immediately for preparing serial dilutions and construction of standard curves.

20S Proteasome

Dilute 5µL of 20S proteasome stock solution (1mg/mL) to 200µL with **Assay Buffer**. Once prepared store **20S Proteasome Working Solution** on ice prior to use and any residual (undiluted) 20S proteasome stock solution at 4°C until required.

Microtitre Plates

White ½-size-well plates for fluorescence are recommended.

Fluorimeter:

Allow the fluorimetric plate reader and associated hardware to warm up and equilibrate. Ensure lamps are on and filters set for excitation at 360nm and emission at 460nm.

Construction of AMC/Substrate standard curves:

For each of the three **AMC/substrate** solutions the following serial dilutions should be prepared in duplicate.

Add **AMC/substrate** working solution (80µL) to well 1.

Add **Substrate/Assay Buffer** solution (40µL) to each of the subsequent 7 wells.

Pipette 40µL from well 1 and mix it thoroughly with contents of well 2.

Pipette 40µL from well 2 and mix it thoroughly with contents of well 3; *etc.*

Discard the last 40µL pipetted from well 8.



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Incubate the plate at 37°C for 30min prior to reading in fluorimetric plate reader.

For all paired samples average the two readings.

Plot graphs of fluorescence units *versus* concentration and calculate the slope for each of the substrates expressed as AFU/ μ M.

For a volume of 40 μ L this gives a calibration factor = average slope/40 in AFU/pmol.

$$\begin{aligned} \text{Factor} &= \text{slope AFU}/\mu\text{M} \\ &= \text{slope AFU}/40\text{pmol (in } 40\mu\text{L)} \\ &= \text{slope}/40 \text{ AFU/pmol} \end{aligned}$$

Proteasome assays:

Set up duplicate assays using 50 μ L **20S Proteasome Working Solution** + 50 μ L **Substrate Solution** in each reaction vial (0.5mL Eppendorf tube). Keep all reactions on ice until all samples are prepared.

Ensure vials are tightly capped and place in 37°C water bath.

After 30min transfer duplicate 40 μ L samples from each vial to individual fluorescence plate wells for assay.

Transfer 40 μ L aliquots of substrate solution to 2 wells for blanks.

Insert plate into fluorimeter and read immediately.

Calculate results as follows:

For all paired samples average the two readings.

Subtract the average blank reading from the average sample reading to obtain ΔF for each sample/substrate pair.

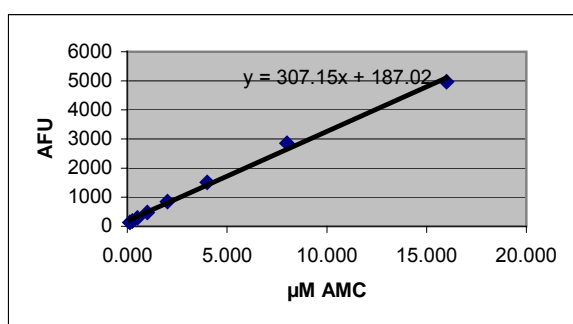
Divide ΔF by AMC-calibration factor (AFU/pmol) obtained from AMC/substrate standard curves to give the amount of AMC liberated at 30 mins.

Divide the above value by 30 to obtain the peptidolytic activity in units of $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ or multiply by two (divide by 30 and multiply by 60) to give the activity in $\text{pmol}\cdot\mu\text{g}^{-1}\cdot\text{hr}^{-1}$.

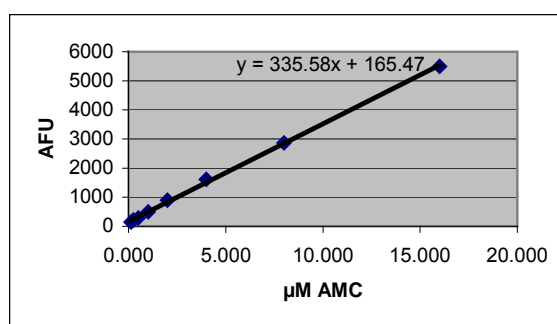
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Example – calibration curves:

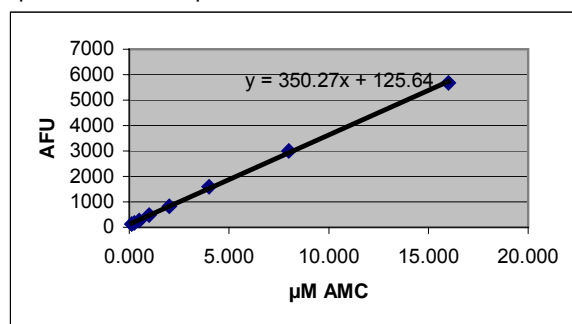
Substrate	Suc-LLVY-AMC			Bz-VGR-AMC			Z-LEE-AMC		
	Fluorescence units			Fluorescence units			Fluorescence units		
$\mu\text{M AMC}$	1	2	Avg	1	2	Avg	1	2	Avg
0.125	137	141	139	143	144	144	132	129	131
0.250	188	185	187	234	191	213	186	178	182
0.500	282	297	290	289	292	291	283	261	272
1.000	480	481	481	491	504	498	505	463	484
2.000	864	838	851	881	924	903	847	805	826
4.000	1503	1525	1514	1640	1586	1613	1654	1546	1600
8.000	2846	2870	2858	2878	2854	2866	2985	3010	2998
16.000	4814	5122	4968	5418	5572	5495	5494	5862	5678



Suc-LLVY-AMC: Factor = slope/40 = 7.68AFU/pmol



Bz-VGR-AMC: Factor = slope/40 = 8.39AFU/pmol



Z-LLE-AMC: Factor = slope/40 = 8.76AFU/pmol

Example – 20S proteasome assay:

Sample	Suc-LLVY-AMC			Bz-VGR-AMC			Z-LEE-AMC		
	1	2	Ave	1	2	Ave	1	2	Ave
Blank	71	86	79	81	82	82	63	64	64
20S	6342	5448	5895	1720	1810	1765	1277	1231	1254
Ave-blank			5817			1684			1191
Factor			7.68			8.39			8.76
Activity (pmol/ $\mu\text{g/hr}$)			1514.7			401.3			271.8