The process of HDAC11 Assay Development: using kit

To monitor the deacetylation activity of HDACs, fluorescence read-out based kits are available. Enzo's Fluor-de-Lys-Green HDAC assay kit (BML-AK530) was used here to monitor the activity of the purified HDAC11. As per the kit, upon deacetylation of the substrate by the HDAC, the developer is able to act on the deacetylated substrate, releasing the fluorophore. This fluorophore is now free of any quenching and thus, its fluorescence is a read-out for the activity of deacetylation by the HDAC. A pictorial representation of the process is illustrated in the Fig 1.

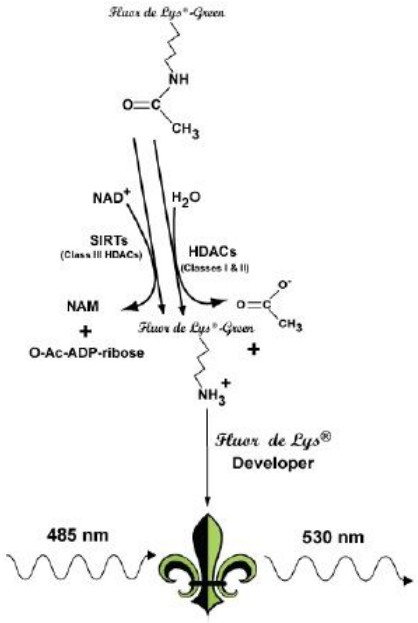

Fig 1. Fluorescence based assay to monitor the deacetylation by HDACs (adapted from Fluor-de-Lys-Green HDAC assay manual, BML-AK530).

Here, as per the instructions of the manual, a preliminary test was performed with the HeLa cell extract (containing HDACs) to confirm if the kit components work appropriately. Table 1 describes the reaction set-up used for this preliminary test.

Table 1. Reaction set-up to monitor the activity of HeLa extract.

25 µl Reaction volume	
Assay buffer (from kit)	50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2
Substrate (kit stock 50 mM)	200 µM, 24 µl (diluted in assay buffer)
HeLa extract (from kit)	1 µl
Reaction time at RT (25 ºC)	0 min, 10 min
25 µl Developer (Diluted to 1X in assay buffer from 20X kit stock), 30 min incubation at RT	

A reaction of HeLa cell extract with the substrate was made for two time points- 0 min and 10 min (this is an end-point assay). A sample with only substrate (no protein being added) was used as a control. After incubation with the developer, for fluorescence measurement 40 µl of the samples were transferred to 384-well black Grenier plates. The excitation and emission filters used with the Synergy4 plate reader were 485/20 nm and 528/20 nm, respectively. For this preliminary assay, the fluorescence was plotted against time in MS-Excel (Fig 2).

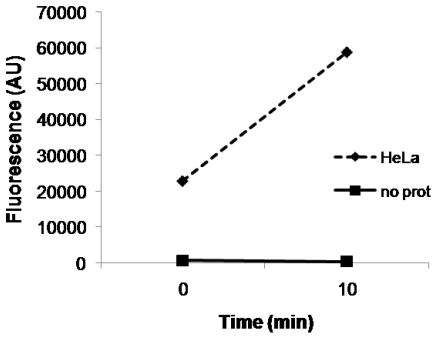

Fig 2. Deacetylation activity of HeLa cell extract.

As compared to 0 min, at 10 min the fluorescence of the sample containing HeLa cell extract increases (but not of the no protein sample/only substrate) confirming the activity of the deacetylases present in the extract (Fig 2). The high fluorescence of HeLa cell extract sample at 0 min suggests the presence of highly active deacetylases in the sample. Hence, the kit components can be used to monitor the activity of our purified HDAC11.

Meanwhile, to reduce the amount of resources, instead of a total 50 µl reaction (25 µl reaction+ 25 µl developer), signals were tested by setting up the reaction with a lower volume (24 µl=12 µl reaction+ 12 µl developer) and instead of 40 µl, 20 µl were transferred onto the black plate for measuring fluorescence. The signals with this lower volume were good enough to proceed.

Further, to monitor the activity of HDAC11, protein purified from *Sf9* was used. As a preliminary test, HDAC11 activity was measured over 1.5 hours at RT. The reactions were prepared using the assay buffer from the kit, as indicated in Table 1. The reaction conditions for HDAC11 are enlisted in Table 2. Fluorescence measurements were made as mentioned above and the data was analyzed using Graphpad.

Table 2. Reaction set-up to monitor the activity of HDAC11.

12 µl Total Reaction volume		
	Stock	Working conc. (µM)
Substrate	50 mM	200
HDAC11	35.6 µM	17.5
Reaction time at RT (25 ºC)	0-90 min	
12 µl Developer, 30 min incubation at RT		

To subtract the background fluorescence, samples with no protein (only substrate) for t=0 min and 90 min were used as blank. In principle, the background fluorescence should remain the same, but here a 66% reduction in blank signal at 90 min in comparison to that at 0 min was observed. Nonetheless, Fig 3

demonstrates that HDAC11 is active (blank signals obtained at 0 min were used to remove the background for all the measurements). In 90 min, the fluorescence signal becomes almost twice when compared to 0 min and the reading at 0 min does not start from 0, which could be an artifact (this preliminary assay here was performed in singlet).

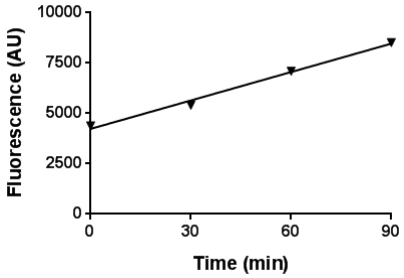

Fig 3. Deacetylation activity of HDAC11.