The process of HDAC11 Assay Development: time-course study

Previously (Fig 1, *data set*), it was shown that HDAC11 appears to be reasonably active with Boc-Lys-(TFA)-AMC substrate. Henceforth, using this substrate, a time-course study was initiated to determine the appropriate enzyme concentration and the time to be used for the reaction (which are essential parameters to perform Km calculations later). For Km calculations, the enzyme concentration and the time chosen should be such that the reaction is in its linear phase. Thus, with this time-course study, the aim was to obtain the complete reaction curve (linear phase followed upon by saturation phase) for each chosen enzyme concentration.

The reaction recipe is enlisted in Table 1. Different enzyme concentrations were used with a fixed (high, non-limiting) concentration of the substrate and the reactions were measured at several time intervals for a period of 90 min. As mentioned previously (*data set*), 10 µl of the final volume (7.5 µl of reaction volume + 7.5 µl of developer solution) was transferred to the 384-well black Grenier plate for measurement with the Synergy4 plate reader at 340/30 nm excitation and 460/40 nm emission wavelengths, respectively. Following a 30 min incubation of the 90 min time point with the developer, the measurements for all the time points were taken simultaneously (as per the Enzo manual which mentions that the fluorescence signal stabilizes after 20-30 min upon the addition of the developer). In addition, for every time point, a blank sample containing no HDAC11 (0 µM) was included.

Table 1. Reaction recipe for time-course study.

7.5 µl Reaction volume	
HDAC11 (µM)	0-2
Boc-Lys-(TFA)-AMC (µM)	200
Assay buffer	20 mM HEPES, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2 0.5 mg/ml BSA (added freshly)
Reaction time at RT (25 ºC)	0-90 min
7.5 µl Developer	
Developer conc. (5X stock)	1X
TSA (Trichostatin A)	40 µM
Incubation time (with respect to last time point)	30 min

The results of this time-course experiment are plotted in Fig 1. The data plotted for each time point is following the subtraction of the background obtained from the corresponding blank samples for each time point. The observations thus, made are:

a. For concentrations of 1-2 µM of HDAC11, there is no increase in activity.
b. For concentrations of 0.125-0.5 µM of HDAC11, there is a linear increase in activity.
c. For concentrations below 0.125 µM of HDAC11, it appears that there is no activity.
d. For all concentrations of HDAC11, the fluorescence signals at time 0 min show activity, instead of starting at zero fluorescence.

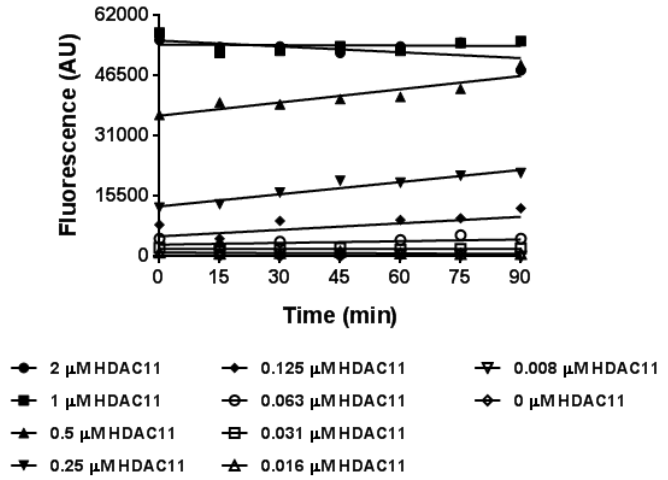

Fig 1. Time-course study for HDAC11.

Since, linearity was achieved for 0.125-0.5 µM of HDAC11 in Fig 1, the above experiment was repeated with a lower conc. range for HDAC (0-0.25 µM). The reaction recipe is enlisted in Table 2.

Table 2. Reaction recipe for time-course study.

7.5 µl Reaction volume	
HDAC11 (µM)	0-0.25
Boc-Lys-(TFA)-AMC (µM)	200
Assay buffer	20 mM HEPES, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2 0.5 mg/ml BSA (added freshly)
Reaction time at RT (25 ºC)	0-120 min
7.5 µl Developer	
Developer conc. (5X stock)	1X
TSA (Trichostatin A)	40 µM
Incubation time (with respect to last time point)	30 min

The results of this time-course experiment are plotted in Fig 2. As earlier, the data plotted for each time point is following the subtraction of the background obtained from the corresponding blank samples for each time point. The observations thus, made are:

a. For concentrations of 250-62.5 nM of HDAC11, there is a linear increase in activity.
b. For concentrations of below 62.5 nM of HDAC11, it appears that there is no activity.
c. For all concentrations of HDAC11, the fluorescence signals at time 0 min show activity, instead of starting at zero fluorescence.

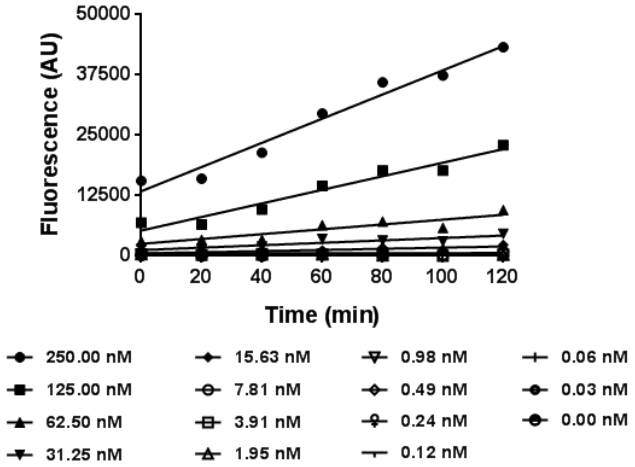

Fig 2. Time-course study for HDAC11.

Analysis of the above observations:

a. Increased fluorescence at 0 min in Fig 1 and 2:
   i. Instead of the reaction producing this fluorescence, the protein might be contributing in this fluorescence.
   ii. The fluorescence signal is not a result of enzyme activity (deacetylation of the substrate followed by the release of AMC), but instead is a result of mere binding of the substrate to HDAC11. In principle, the conjugation (vicinity) of AMC to amino acids leads to quenching of its fluorescence, thus, this reason looks unlikely.
   iii. HDAC11 exhibits pre-steady state kinetics.
b. No activity for HDAC11<0.125 µM in Fig 1 and for HDAC11<62.5 nM in Fig 2:
   i. HDAC11 conc. is too low to show any activity in the 90 min/120 min period used.
   ii. Due to the scale used in the plot, the increase in fluorescence signal is not visible.
c. No activity for HDAC11>0.5 µM in Fig 1:
   i. HDAC11 becomes inactive at higher conc.
   ii. All the substrate has been catalyzed by HDAC11 at time 0min itself.
   iii. The signals are above the detection limit for the instrument (checked and thus, this reason was discarded).