# The process of HDAC11 Assay Development: linearity and Km calculation

So far in the process of optimization for assay development, 0.125 µM HDAC11 and 200 µM Boc-Lys-(TFA)-AMC have been used with a reaction time of 30 min to start off with. But to calculate the Km of the enzyme, the optimized buffer conditions should be used first to find out the concentration of the enzyme and the corresponding appropriate reaction time that gives a linear curve with an appropriate signal.

## Linear kinetics of HDAC11

To obtain this linearity, a time-course activity measurement was performed with different concentrations of HDAC11. The recipe for the reaction is described in Table 1.

**Table 1.** Reaction recipe for enzyme linearity.

| 7.5 µl Reaction volume | |
|---|---|
| HDAC11 (µM) | 0.06-1 µM |
| Boc-Lys-(TFA)-AMC (µM) | 200 |
| Assay buffer | 20 mM Bicine, pH 8.5,<br>137 mM NaCl,<br>2.7 mM KCl,<br>1 mM MgCl2<br>0.5% BSA (added freshly) |
| Reaction time at RT (25 ºC) | 0-6 hours |
| 7.5 µl Developer | |
| Developer conc. (5X stock) | 0.1 X |
| Incubation time | 1 hour |

Fig 1A shows the activity of HDAC11 at varying concentrations of the enzyme over a period of 6 hours. For the initial phase of the enzyme reactions, measurements were taken every 5 min. Gradually, the time interval was increased. The plots from Fig 1A are zoomed in and represented for the initial 30 min in Fig 1B and 1C. Fig 1C is a further zoomed in representation of Fig 1B, showing the linear curves for lower HDAC11 concentrations. Here in Fig 1A, the curves are fitted using Graphpad as per the Michaelis-Menton equation upon subtraction of the blank (only substrate measured for each time point) to give an overview of the saturation phase of the enzyme reaction. Since, HDAC11 has been showing burst kinetics, the linear curves represented in Fig 1B and 1C, show the burst and thus, are fitted accordingly.

Observations:

1. As per the 6 hour assay, at higher concentrations 0.25 µM and higher, the reactions tend to plateau at similar values of fluorescence. The lower concentrations of HDAC11 on the other hand, are still catalyzing the substrate.
2. The reactions are linear for all the enzyme concentrations over the initial 30 min of the assay.
3. The signal to noise ratio is very low over the 30 min reaction time for concentrations $\leq 0.125$ µM.

Inferences:

1. Since the reactions plateau at the same level, the enzyme is active over the time-scale used for the measurements.
2. To avoid using very high concentration of HDAC11 for the purpose of screening, in order to maintain the balance between the enzyme concentration and the best possible signal to noise ratio, the linearity of 0.25 µM of HDAC11 could be used.

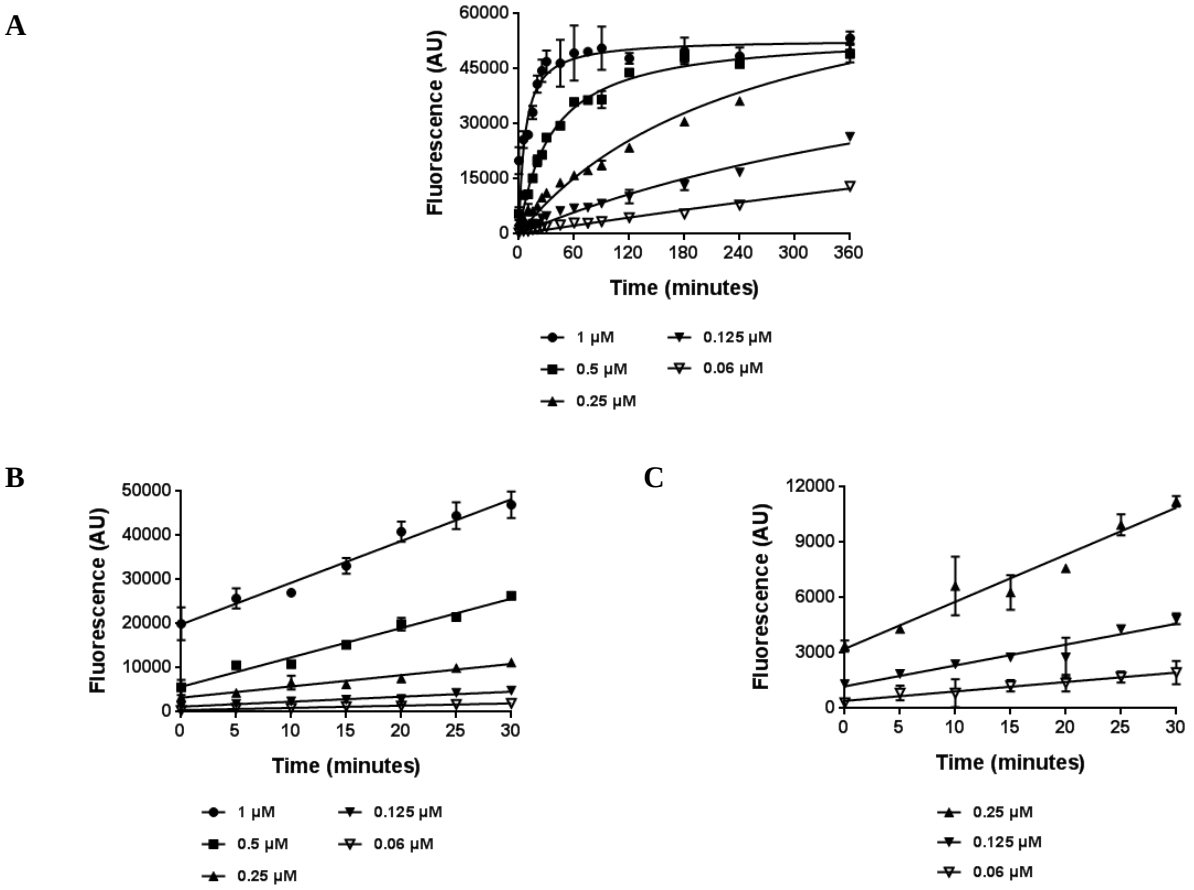


**Fig 1.** Time-course assay of HDAC11. (A) Activity of HDAC11 at different enzyme concentrations over a period of 6 hours. (B) and (C) Activity of HDAC11 at different enzyme concentrations over a period of 30 min.

Henceforth, in order to calculate the Km (the substrate concentration), 0.25 µM of HDAC11 will be assayed for a 30 min reaction time.

## Km calculation

In order to calculate the Km of the substrate, Boc-Lys-(TFA)-AMC with HDAC11, activity of HDAC11 was measured for 30 min (linear range as obtained from above) at different substrate concentrations. The recipe for the reaction is described in Table 2.

**Table 2.** Reaction recipe for Km calculation.

| 7.5 µl Reaction volume | |
|---|---|
| HDAC11 (µM) | 0.25 µM |
| Boc-Lys-(TFA)-AMC (µM) | 0-250 µM |
| Assay buffer | 20 mM Bicine, pH 8.5,<br>137 mM NaCl,<br>2.7 mM KCl,<br>1 mM MgCl2<br>0.5% BSA (added freshly) |
| Reaction time at RT (25 ºC) | 0-30 min |
| 7.5 µl Developer | |
| Developer conc. (5X stock) | 0.1 X |
| Incubation time | 1 hour |

Fig 2A shows the plot of HDAC11 activity over a 30 min period at varying concentrations of the substrate. All the concentrations used here, produced linear curves for enzyme activity under the assay conditions. The slopes of all of these were calculated and plotted against the substrate concentration, as shown in Fig 2B. The curve was fitted using Michaelis-Menton equation in Graphpad and the km was calculated to be 30.17 ± 6.70 µM.

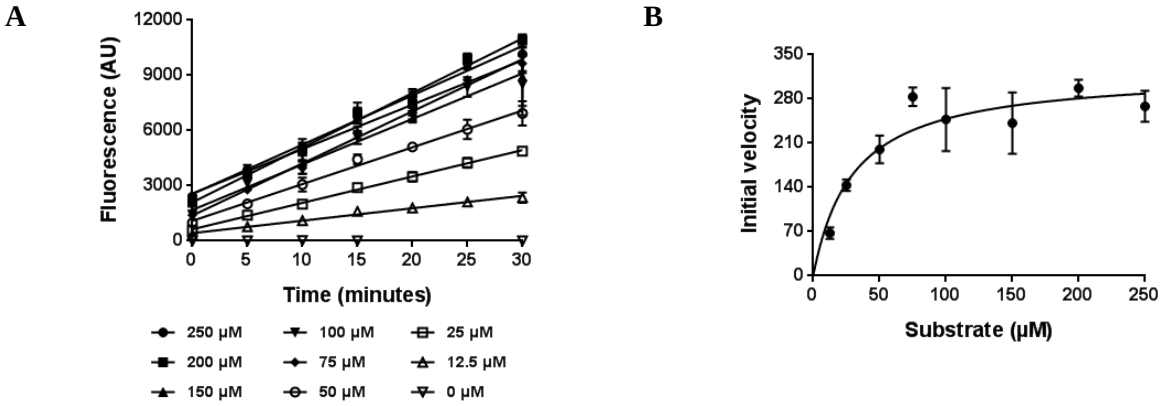


**Fig 2.** HDAC11 kinetics to calculate the Km for its substrate, Boc-Lys-(TFA)-AMC. (A) Time-course measurement for HDAC11 activity at varying substrate concentrations over a period of 30 min. (B) Plot of initial velocities (slopes) corresponding to each substrate concentration in order to obtain the Km upon fitting with Michaelis-Mention equation (using Graphpad).

Further optimization (if required) and screening should thus be performed using 0.25 µM of HDAC11and 30 µM of substrate with a reaction time of 30 min.