The process of HDAC11 Assay Development: Check for linearity and Km-repeat

As performed earlier (*dataset*), the linearity for HDAC11 activity for Boc-Lys-(TFA)-AMC substrate was monitored again using the newly optimized buffer conditions. This was followed upon by Km calculation.

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)	100
Assay buffer	20 mM Tris, pH 7.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2 0.5 mg/ml BSA (added freshly) 5 mM TCEP
Reaction time at RT (25 ºC)	0-5 hours
7.5 µl Developer	
Developer conc. (5X stock)	0.1 X
Trichostatin A (TSA)	40 µM
Incubation time	1 hour

Fig 1A shows the activity of HDAC11 at varying concentrations of the enzyme over a period of 5 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. 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, show the burst and thus, are fitted accordingly.

Observations:

1. As per the 5 hour assay, for HDAC11 $\geq$ 0.5 µM, the reactions reach saturation; for HDAC11 = 0.25 µM, the reaction approaches saturation, while for HDAC11 $\leq$ 0.125 µM, reactions are still in the process of catalyzing the substrate.
2. The reactions are linear for HDAC11 $\leq$ 0.25 µM over the initial 30 min of the assay.

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 will be used for 30 min period to calculate Km and for screenings further on.

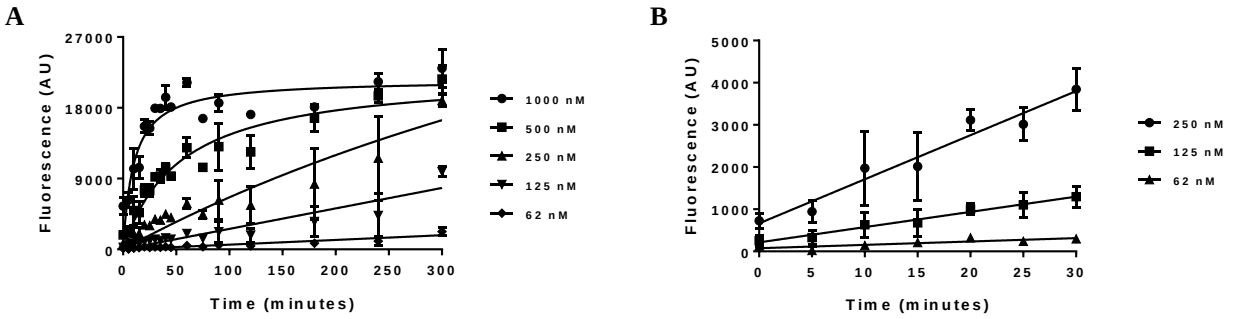

Fig 1. Time-course assay of HDAC11. (A) Activity of HDAC11 at different enzyme concentrations over a period of 5 hours. (B) Activity of HDAC11 at different enzyme concentrations over a period of 30 min.

Km calculation

In order to calculate the Km for 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.

Fig 2A-H 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 2I. The curve was fitted using Michaelis-Menton equation in Graphpad and the Km was calculated to be 48 ± 17 µM.

Table 2. Reaction recipe for Km calculation.

7.5 µl Reaction volume	
HDAC11 (µM)	0.25 µM
Boc-Lys-(TFA)-AMC (µM)	0-150 µM
Assay buffer	20 mM Tris, pH 7.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2 0.5 mg/ml BSA (added freshly) 5 mM TCEP
Reaction time at RT (25 ºC)	0-30 min
7.5 µl Developer	
Developer conc. (5X stock)	0.1 X
Trichostatin A (TSA)	40 µM
Incubation time	1 hour

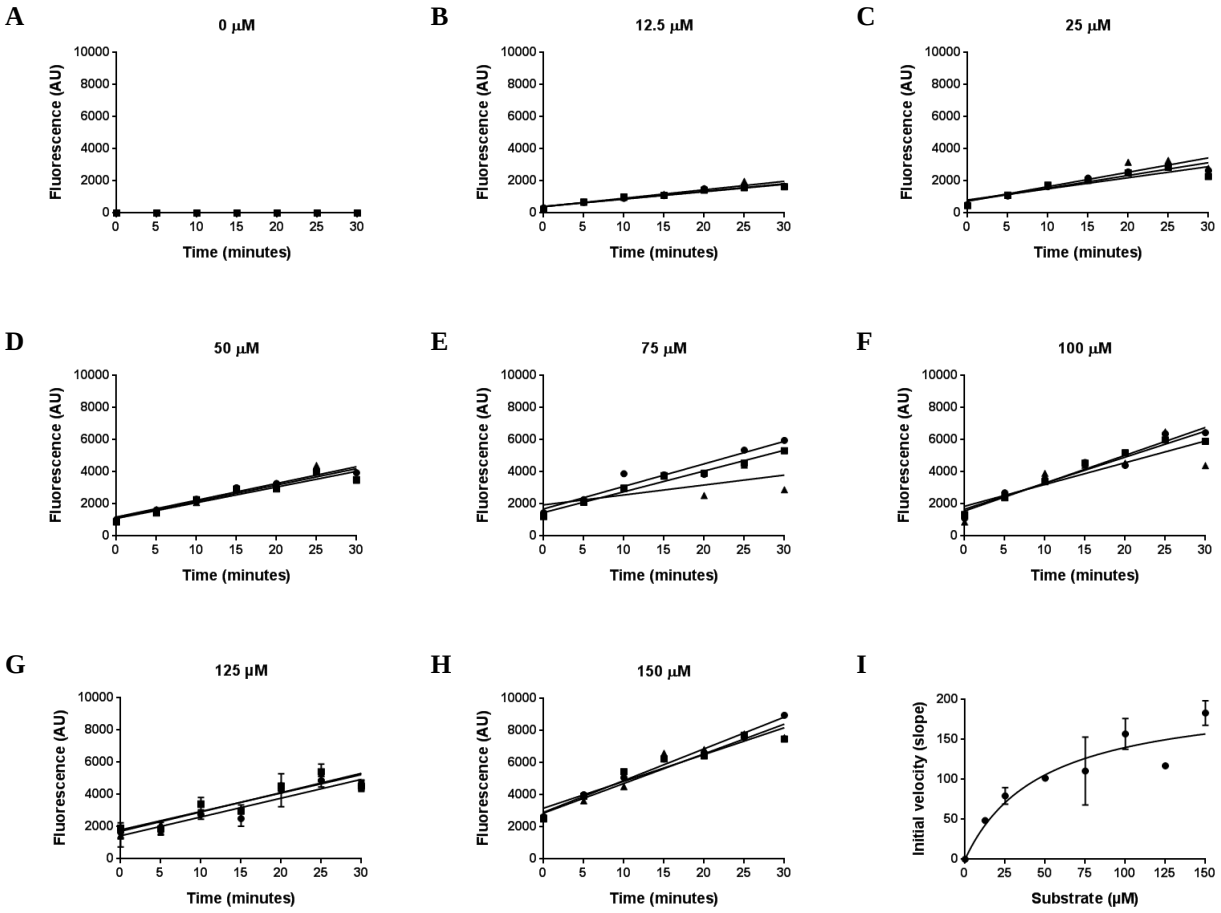

Fig 2. HDAC11 kinetics to calculate the Km for its substrate, Boc-Lys-(TFA)-AMC. Time-course measurement for HDAC11 activity at (A) 0, (B) 12.5, (C) 25, (D) 50, (E) 75, (F) 100, (G) 125 and (H) 150 µM of Boc-Lys-(TFA)-AMC substrate over a period of 30 min. (I) Plot of initial velocities (slopes) corresponding to each substrate concentration in order to obtain the Km (48 ± 17 µM) upon fitting with Michaelis-Mention equation (using Graphpad).

Further optimization (if required) and screening should thus, be performed using 0.25 µM of HDAC11 and 48 µM of substrate with a reaction time of 30 min.