HTS case study: Comparison of endpoint and kinetic reads

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1 INTRODUCTION

In collaboration with our Client we have developed a Fluorescence Polarisation (FP) assay using BellBrook Labs Transcreener® UDP^2 Assay kit in both endpoint and kinetic read formats for high throughput screen ("HTS") purposes to identify small molecule inhibitors of our Target (**Fig 1**). An orthogonal biochemical colorimetric assay was also developed by our Client for screening purposes. We assessed both formats in a pilot screen and the FP format was selected based on robustness for HTS purposes, with the other format being used as an orthogonal assay in follow up potency studies. The FP assay was used to screen approximately 300,000 Discovery UK library compounds comprising of the entire Discovery UK Lead-Like Library of approximately 155,500 compounds together with 150,000 compounds selected by the Client using a plate-based selection from the Discovery UK Diversity Library of 625,000 compounds.

Fig 2. Biocel fully automated platform

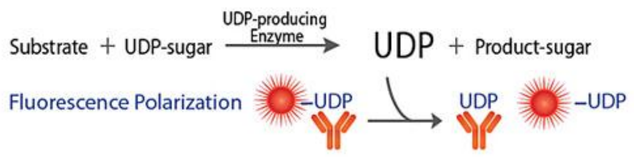

Fig 1. Assay schematic of the Transcreener® UDP^2 FP Assay kit from Bellbrook labs

The Biocel fully automated platform **(Fig 2**) was employed to enable the HTS assays to be run in kinetic mode. Performing the assays in kinetic mode enabled analysis of the compound effect by both calculation of slope and endpoint readings. A kinetic read has a significant advantage over an endpoint reading, as is common in HTS programs, in terms of identifying false positives and interfering compounds. A comparison of kinetic slope analysis and endpoint readings will be discussed highlighting common pitfalls in assessing the activity of compounds.

2 EXPERIMENTAL

The primary FP assay was optimised for HTS purposes and performed in black 384-well plates and typical reaction time course data is shown in **Fig 3**. Compounds were dispensed using the Echo acoustic dispensing platform and incubated with the enzyme in a buffer containing 50 mM Tris pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 0.2mg/ ml BSA and 2% DMSO. The substrate was added to start the reaction and plates were read kinetically over the linear phase of the reaction using the BMG Pherastar for 10 mins at read intervals of two minutes. The final time point provided the data for the end point assay reading. Mars software within the Pherastar was used to calculate slopes between specified time points.

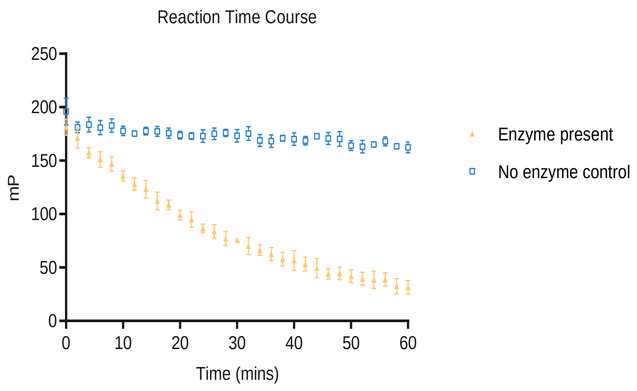

Fig 3. Transcreener® UDP^2 FP reaction time course in presence and absence of enzyme

Data was analysed using Genedata Screener and was normalised against a positive control to determine percentage activity data for each compound. Genedata heatmaps were inspected visually to identify any unusual plate effects. Plates were passed on criteria of Z' Factor > 0.5 and signal window > 3 for both assay formats.

3 SCREENING RESULTS

A pilot screen of 10,000 compounds tested in duplicate in the FP and orthogonal assay formats was performed, the correlation of the replicates obtained in the FP assay is shown in **Fig 4**. Both assays produced a robust assay with acceptable plate statistics with Z' >0.5, typical run data for the FP assay is shown in **Fig 5**. Following the successful pilot screens the primary HTS was performed in the FP format to screen the remaining 290,000 compounds. Each compound was tested at a final concentration of 20 μM. Following analysis of the HTS data 3,088 compounds were selected as potential hit compounds to be confirmed in both the FP and orthogonal assay formats in duplicate at a single concentration. Subsequently 320 compounds were selected to be tested for potency determination as 10pt. concentration response curves in duplicate in both assay formats.

Fig 4 Normalised 0-10 minutes slope FP assay data correlation of N=1 and N=2 data.

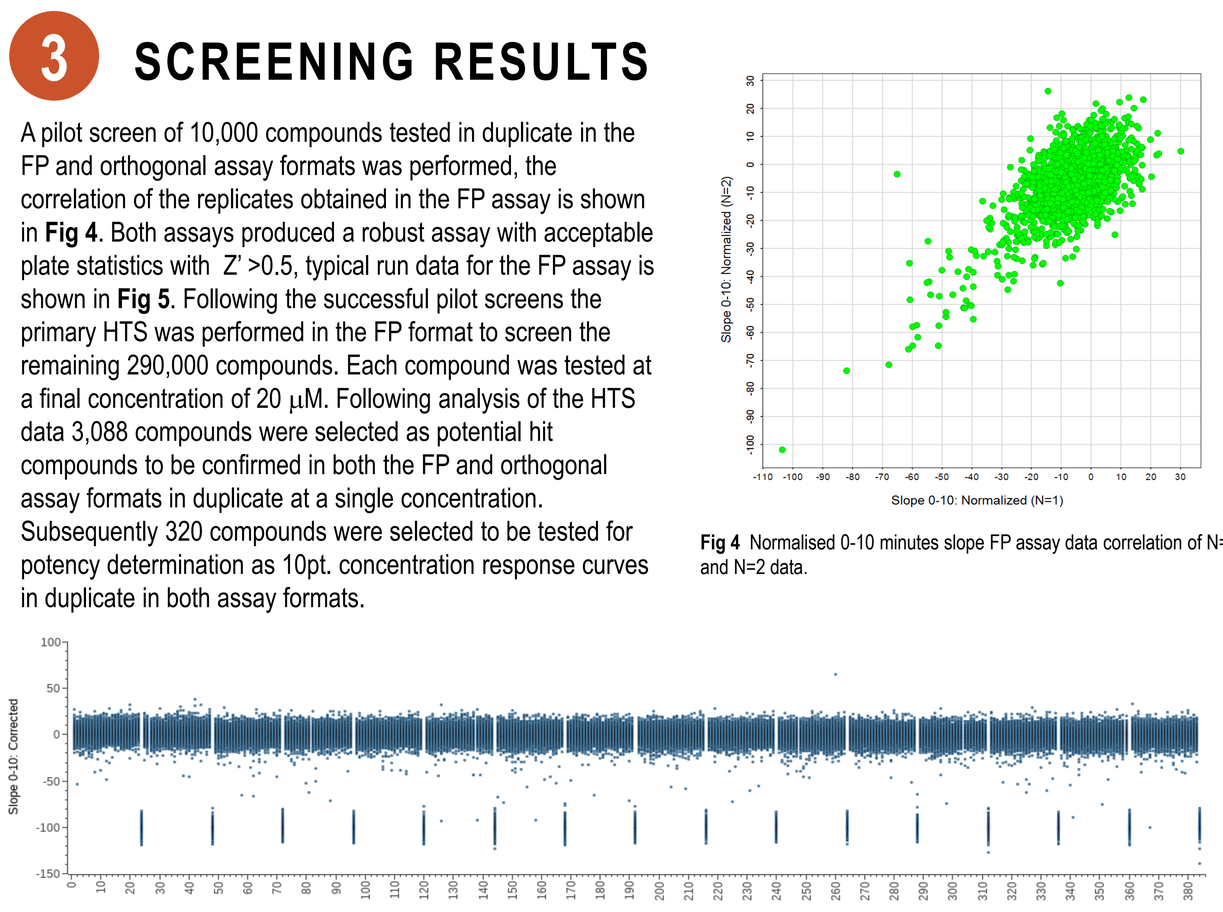

Fig 5 . Corrected 0-10 min slope well data across a typical run

4 COMPARISON OF KINETIC AND ENDPOINT READS

The Biocel fully automated platform enabled the HTS assays to be run in kinetic mode at all phases of the screening workflow. In the potency phase of the screening cascade 10pt. concentration response curves in duplicate in both assay formats were performed and analysed using both endpoint and calculated slope data at each compound concentration to determine the activity of the compound. The examples below highlight the importance of analysing the raw kinetic data to enable accurate assessment of the activity of the compounds.

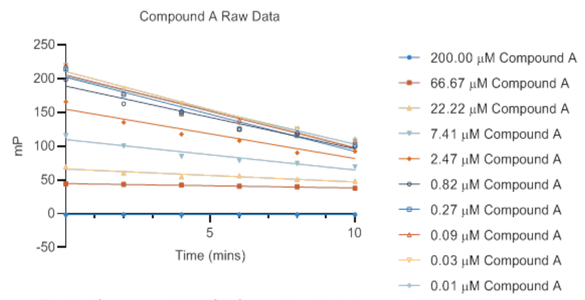

Fig 6 . Compound "A" CRC raw kinetic data

The raw kinetic data over the linear 10 minute time course for compound A in the FP assay is shown in **Fig 6**. When the 10 minute endpoint readings are normalised and plotted the compound appears to behave as an activator of the enzyme.(**Fig 7a**). In contrast when the slope data are normalised and plotted this results in the compound appearing as an enzyme inhibitor (**Fig 7b**). Analysis of the raw kinetic data suggest that compound A is interfering with the readout leading to misleading and conflicting results between the normalised endpoint and kinetic slope data.

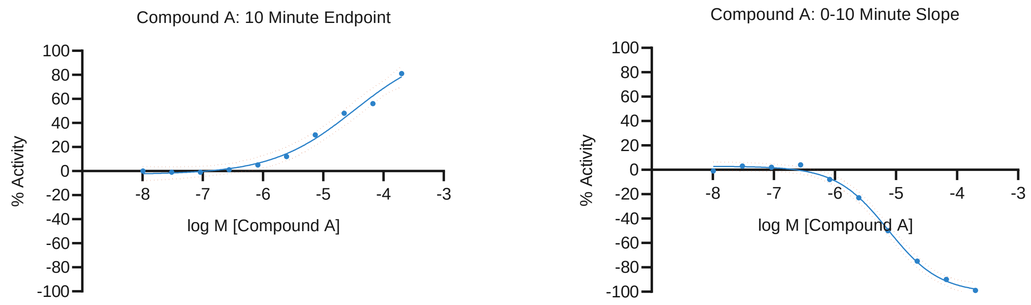

Fig 7. Compound "A" CRC curves generated using **a.** Endpoint read (10 minutes incubation) method and **b.** Slope between 0-10 minutes method.

In the orthogonal assay reaction linearity was longer than in the FP assay so a 20 min reaction time point was used. The raw kinetic data over a linear 20 minute time course for compound B is shown in **Fig 8** for the orthogonal assay format. When the 20 minute endpoint readings are normalised and plotted the data appears to suggest the compound is inhibiting the enzyme.(**Fig 9a**). In contrast when the slope data are normalised and plotted, the compound appears to be inactive (**Fig 9b**). Analysis of the raw kinetic data suggest that this compound is interfering with the readout leading to a false positive result when analysing the endpoint data. Analysis of the raw kinetic data indicates that compound B acts to increase the absorbance readout in a concentration dependent manner while having no effect on the slope.

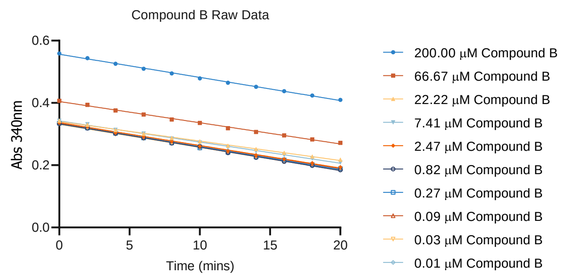

Fig 8 . Compound "B" CRC raw kinetic data

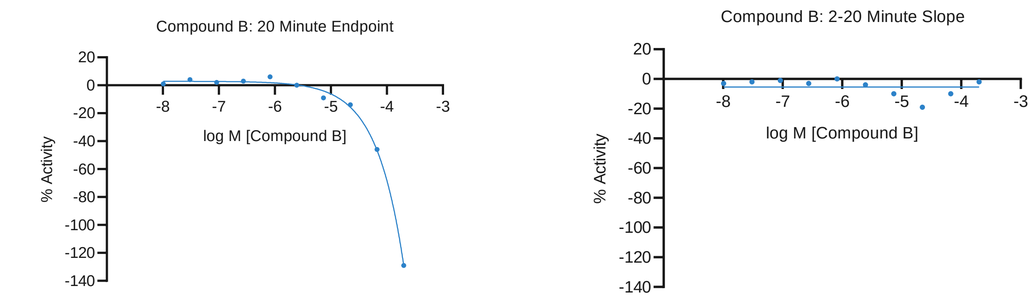

Fig 9 . Compound "B" CRC curves generated using **a.** Endpoint read (20 minutes incubation) method and **b.** Slope between 2-20 minutes method.

5 CONCLUSIONS

In collaboration with our client we have successfully developed a high-throughput FP assay to screen approx. 300,000 compounds against the chosen target. The primary FP assay and orthogonal assay were both performed in kinetic mode in a high-throughput format enabled by using the Biocel automated platform. We have highlighted the advantages of performing a kinetic read and the importance of analysing the raw kinetic data to correctly establish the activity of the compounds. The two examples given have shown the importance of analysis of the raw kinetic data to ensure that the interpreted results from the normalised kinetic or endpoint data is not misleading or result in conflicting conclusions. Also due to the nature of fluorescent assay technologies it is advisable to also ensure that compound activity is also confirmed in an orthogonal assay format before progressing further. This is invaluable in terms of identifying false positives or assay interfering compounds over a traditional endpoint only read assay before progressing compounds through the drug discovery cascade.

6 ACKNOWLEDGEMENTS

Thank you to our Client for giving their permission to use the data from our collaboration. Also to the Charles River compound management team for supply of compounds for the screening activities.