Use of TR-FRET in HTS to identify small molecule stabilisers of a temperature sensitive protein

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1 ABSTRACT

Protein denaturation is a key process controlling protein activity. Certain mutations can de-stabilise proteins making them more susceptible to denature due to factors such as an increase in temperature, leading to an abnormal or non-functional protein. Therefore small molecule correctors that stabilize the native structure of the protein at high temperatures are of interest. Thermal shift assays are widely used in hit-to-lead projects where the temperature at which proteins "melt" in the presence and absence of test compounds can be determined but this format is low throughput and not amenable to high throughput screening (HTS). In this project we have utilized Time-Resolved Fluorescence Energy Transfer (TR-FRET) to monitor a temperature-induced conformational change in the protein to enable a HTS to identify small molecule correctors of the target protein. Interestingly when exploiting thermal stabilisation assay for the purpose of HTS many challenges were encountered, particularly as the assay was scaled-up, and needed to be overcome if throughput was to be achieved.
Here we highlight Charles River's approach to successfully execute a challenging 155,000 compound HTS campaign using a thermal stabilization as an assay readout resulting in the identification of novel chemical matter for the target class under investigation by our Partner.

2 INTRODUCTION

Thermal shift assays are used to assess changes in protein melting temperature in the presence and absence of test compound[1]. However this assay format is low throughput and therefore not amenable to HTS. At Charles River Laboratories, the principles of thermal shift assays were combined with TR-FRET detection to identify small molecule corrector compounds that bound and stabilised a temperature sensitive, mutant form of the target protein during a HTS campaign. The assay was based on the premise that the temperature sensitive, mutant protein would undergo an irreversible conformational change at high temperatures due to a decrease in thermo-stability compared to its wild type counterpart.
TR-FRET detection is a well-established and robust technology frequently used in HTS, providing a highly-sensitive and reproducible platform even in high density microplate formats[2], achieving assay throughputs suitable for HTS purposes.
The HTS aimed to identify small molecule corrector compounds that thermo-stabilised the mutant protein, preventing the protein from irreversibly denaturing at 32°C, resulting in restoration of normal protein biological function. The non-denatured conformation of the protein is detected in the assay by an increase in the TR-FRET signal as the Europium bound antibody specifically binds to the stabilised conformation and hence in close proximity with APC labelled antibody.

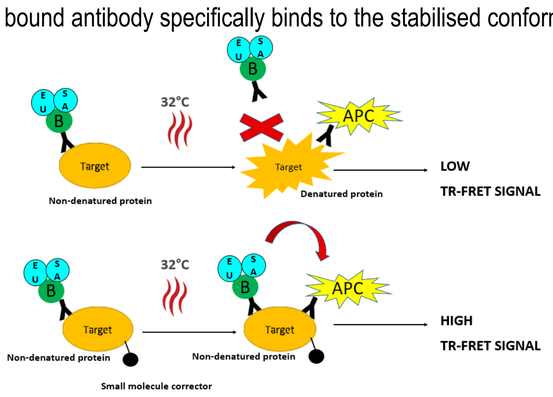

Figure 1. TR-FRET assay readout allowed detection in signal changes related for quantitative measurement of non-denatured and denatured forms of the mutant protein target

The TR-FRET assay to measure protein conformational changes of a temperature sensitive mutant protein presented here was optimised into a robust assay amenable to HTS. As the assay was adapted for the screening of large number of compounds we encountered and overcame some unexpected manual and logistical challenges. We discuss how these were manifested in the data obtained and the approaches used to address these to allow execution of a 155,000 compound HTS. As a result, novel chemical matter for the target class under investigation has been identified.

3 MATERIALS AND METHODS

Assay reagents and assay protocol were supplied by our Partner. The initial protocol was modified and successfully miniaturized to minimize reagent consumption and enable an increase in assay throughput. The assay was optimized and validated into a HTS amenable format using automation prior to testing of compound libraries (Figure 2). Compounds were pre-dispensed into 384-well assay plates using acoustic dispenser (Echo555® Access system, Labcyte™). We also attempted to further miniaturize the assay in 1,536-well plate format, however this was unsuccessful due to uneven signal across the plate, compromising assay robustness.
Charles River's 155,000 compound Lead-like library was selected for screening in order to maintain selectivity for drug-like compounds.
Datasets were analysed using Genedata Analyzer™ software, with TR-FRET ratio signal normalized to % activity calculated from values obtained for the denatured protein in the absence of any test compounds.

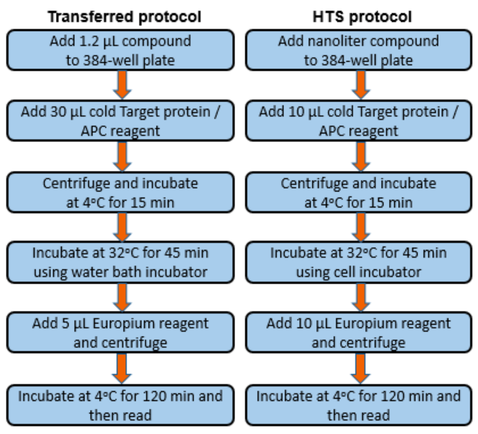

Figure 2. Comparison between the protocol transferred from our Partner and optimized assay workflow for the HTS.

4 ASSAY SENSITIVITY AND TECHNICAL CHALLENGES DURING SCALE UP FOR HTS

A number of challenges were encountered and overcome prior to the initiation of the HTS and during assay scale up. These are outlined below with supporting data shown in Figures 3 and 4.

- Marked plate effects observed in 384-well format indicating uneven heating of plates when transferred into the 32°C incubator from 4°C (Figure 3). Increasing the speed of heat transfer across the plate was found to be the key aspect for improving plate uniformity. This was achieved through increasing the contact area of metal to the plates by using metal shelves instead metal stackers within the incubator.
- Additionally, plates were evenly distributed across the incubator metal shelves, and was critical for uniform heat distribution.
- Faster plate cooling after transfer from 32°C incubator back into 4°C fridge after Europium addition was essential and achieved by stacking plates no higher than four plates with sufficient gaps between stacks.
- Genedata correction function was applied in order to correct any remaining minor plate effects.
- Source and grade of BSA used in the assay affected activity of the reference compound and also overall assay performance.
- BSA from supplier 4 resulted in significant increase in reference compound response comparable to initial results during assay transfer phase (Figure 4).
- Multiple parameters including reference compound pharmacology, assay noise and plate statistics were assessed to ensure the assay was sufficiently robust and sensitive before initiating the HTS.

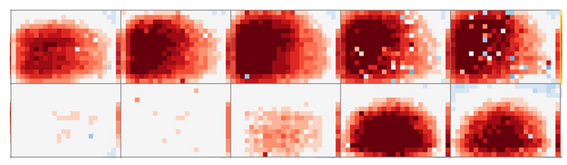

Figure 3 Marked plate effects observed during the transfer of bulk quantities of 384 well plates to the incubators. Heat map scale representing 100% activation dark red to -100% activation dark blue

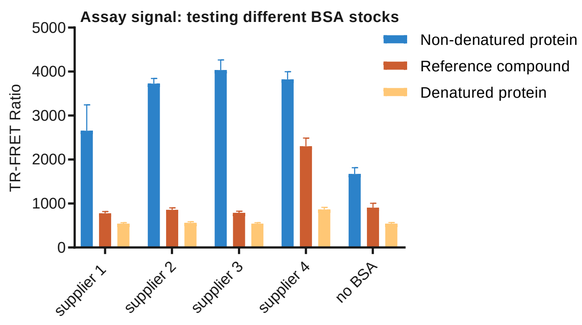

Figure 4 The quality of BSA affecting reference compound activity and overall assay performance

5 RESULTS AND DISCUSSION

A biochemical TR-FRET assay measuring protein conformational changes was successfully used to screen 155,000 compounds using a 384-well plate format over 10 screening days. A modest throughput of 50 plates per day was achieved in order to maintain consistent assay performance and sensitivity throughout the HTS.

- Minimal plate effects of <20% activation for the majority of plates observed; Genedata correction improved minor plate effects observed in the datasets.
- Well level data for all assay controls and test compounds were tracked during the HTS (Figure 4).
  - Reference compound controls resulted in the expected % activation.
  - Non-denatured mutant protein control was added after the 32°C incubation step to assess maximum possible compound % activation. Due to addition after the 32°C incubation step, the non-denatured mutant protein control did not track with the denatured mutant protein control assay signal as expected.
- Low assay noise observed across the entire HTS enabling detection of weak active compounds.
- Successfully identified hits that were progressed to later stages of the project.

Total of 1,121 HTS hits selected using a 20% normalised data and 15% corrected data cut-off representing 0.7% hit rate, in line with expectation considering the biology of the target under investigation.
303 compounds confirmed as hits on duplicates tested and were progressed to the potency determination stage based on >20% confirmed activation or 15-20% activation and part of an active compound cluster.
Good correlation for the potency determination stage (Figure 5) was obtained. Curves were fitted to compounds demonstrating >25% maximum activation. Majority of compounds tested, 218 / 303, demonstrated an EC_{50} <maximum test concentration of 100 µM.

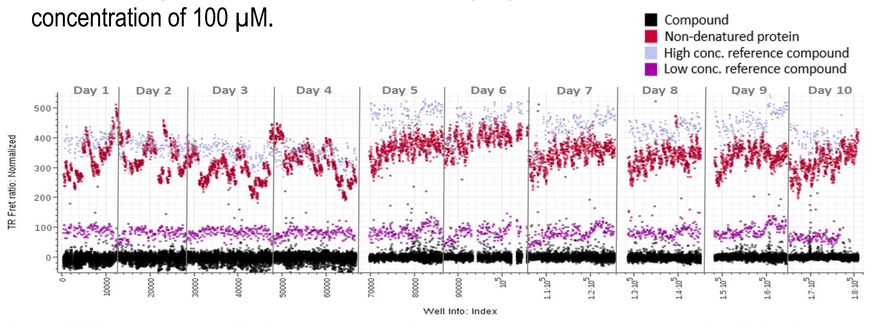

Figure 4 HTS data normalised to % of activation showing reference compound activity at two concentrations (full stabilisation and partial stabilisation) for multiple screening. The data analysis was performed with Genedata software. Very low assay noise across the HTS enabled a good separation of hits from the inactive compounds resulting in a reasonable hit rate for this target.

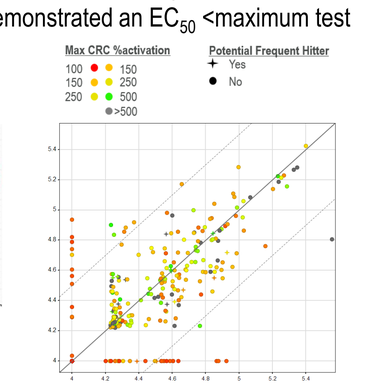

Figure 5. Data correlation for the compound potency determination for two separate runs.

6 SUMMARY AND CONCLUSIONS

- **Low throughput TR-FRET assay measuring protein conformational changes was successfully transferred and optimised for HTS purposes in 384-well format.**
- **Unexpected issues during assay scale up were successfully overcome to achieve assay throughput of 50 plates per day and subsequent completion of a 155,000 compound screen.**
- **218 compounds identified with measurable EC_{50} < 100 µM.**
- **This screen highlights Charles River's capabilities to execute a challenging TR-FRET biochemical HTS assessing conformational change of a temperature sensitive mutant protein enabling the identification of novel small molecule corrector compounds that can be used as a starting point for Structure Activity Relationship studies.**

7 ACKNOWLEDGEMENTS

We would like to acknowledge Cepter Biopartners as the company who developed the assay for our Partner.

8 REFERENCES

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