Challenging Cellular HTS to Identify Small Molecule Upregulators of a Critical Biological Process with the Target Driven Biosensor Readout

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1 ABSTRACT

High Throughput Screening (HTS) using inventively designed cellular models can provide a high volume of data on the activity of a large number chemical compounds, their potential mechanism of action and can differentiate biological activity from unwanted toxicity. The continuous advancement of technology in this field allows the development of sensitive, reliable and robust phenotypic cellular assays to triage active compounds from large screening libraries.
We outline a case study for a challenging cellular HTS campaign that was successfully completed and resulted in the identification of diverse chemical matter to support the early drug discovery process.
We present the approach for transitioning an existing complex cellular assay to allow successful execution of a ~500,000 compound screen. We also present the strategies we used to overcome the scientific and technical challenges faced during the HTS including data handling and the hit calling approach.

2 INTRODUCTON

In comparison with simplified and homogeneous biochemical screens, cell based HTS frequently present significant technical challenges. Moreover, the use of stable cell lines expressing the protein of interest can add to these complications due to variations in the expression levels depending on the induction threshold and loss of expression after multiple cell passages.
In this screen, we were looking for a small molecule to induce expression of a protein involved in a biological process and consequently associated with cell survival. Maintaining low levels of expression prior to the assay was therefore crucial to avoid cell lethality. The assay principle relied on establishing a baseline of low but non-lethal amounts of protein and measuring induction of the pathway (i.e. production of new protein) following compound treatment using a Nano-BiT® complementation method (Figure 1). A pre-requisite for robust assay performance was to strike a balance between the basal protein levels and cell health.

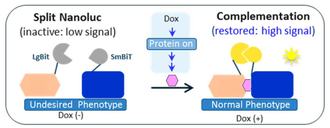


Figure 1. Nano-BiT® based biosensor allowed detection of changes in signal related to the degree of pathway induction

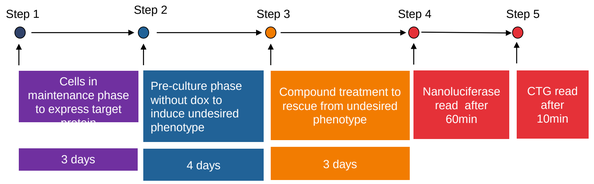
3 MATERIALS AND INSTRUMENTS

Human embryotic kidney cells (HEK-293) were supplied to Charles River by our Partner, expanded and frozen stocks prepared for HTS purposes. HEK-293 cells were CRISPR knock out for the wild type copy of the target protein and replaced with a Doxycycline inducible mouse isoform to avoid cell lethality. Cells were further modified to express the Nano-BiT® based biosensor (Promega) for live cell monitoring of the biological process under investigation. The assay was multiplexed with CellTiter-Glo® (CTG) to measure cell viability. The assays were performed in 384-well white, cell culture treated plates (Perkin Elmer) and luminescence was measured on Envision plate reader. For HTS purposes, cells were added directly to acoustically dispensed assay ready compound plates (Echo555 Access system, Labcyte). Detection substrates were purchased from Promega.

4 WORKFLOW OPTIMISATION FOR HTS

Multiple factors affected the robustness and consistency of the assay and the workflow required significant optimization.
These factors included:

- Initial expression levels of the target protein which was driven by concentration and time of doxycycline exposure
- Time of pre-culture phase allowing reduction in target protein levels for measurement of a robust inducible effect on the biological process
- Cell growth rates during above two phases
- Compound exposure time required to restore the biological process whilst causing minimal toxicity and maintaining assay sensitivity



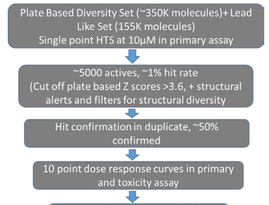


Figure 2. (a) Essential balance between maintenance, pre-culture and compound exposure times was achieved with the described workflow. (b) Flowchart showing the scale of HTS, number of compounds screened and hit selection criteria

5 ASSAY SENSITIVITY AND TECHNICAL CHALLENGES DURING SCALE UP FOR HTS

Additional technical challenges were encountered and overcome during the scale up and batch testing ahead of HTS, including:

- Relative instability of the expression system
  - Cells between passages 4 and 8 were required for robust signal
- Temperature sensitivity of the Nanoluc reagent
  - The detection step had to be carried out at 37ºC due to protein complementation
  - Batch size was limited to 10 plates with up to 10 batches of plates processed in a 1 day
- Temperature fluctuation observed between incubators affecting assay signal
  - It was necessary to select incubators with better airflow and stable temperatures
  - Fan position within incubator (rear or top mounted) made a difference

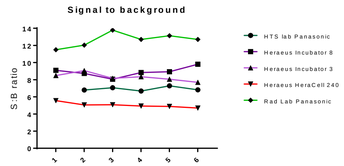


Figure 3. Assay plates from single experiment indicating significant variations in signal to background with single parameter change i.e. change in the incubator

6 RESULTS AND DISCUSSION

The cellular assay was used to screen approximately 500,000 compounds (384 well plates) with a consistently acceptable performance based on robust Z'-factor >0.5

- Multiple parameters and long incubation times introduced variability in the data requiring compensation during hit calling
- Significant number of compounds interfered with detection reagents
- Number of compounds with high potency relative to the reference compound
- Successfully identified hit compounds with activity that confirmed in the screening cascade

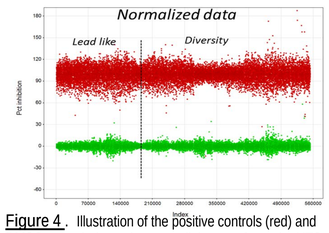


Figure 4. Illustration of the positive controls (red) and DMSO controls (green) across all the runs from the HTS

6 RESULTS AND DISCUSSION CONTINUED…

The distribution of active compounds was found to be uniform across all the HTS runs based upon the hit rate. Data was analyzed with Genedata software and a hit calling approach based on the use of robust Z-score (RZ-score) was used to take account of the variability seen between HTS runs. The normalized percent control data relative to DMSO and positive control wells on each plate was used as a criteria to understand biological activity of compounds in the assay. Genedata's correction function was applied to the normalized data to mitigate any positional effects on the distribution of hits.

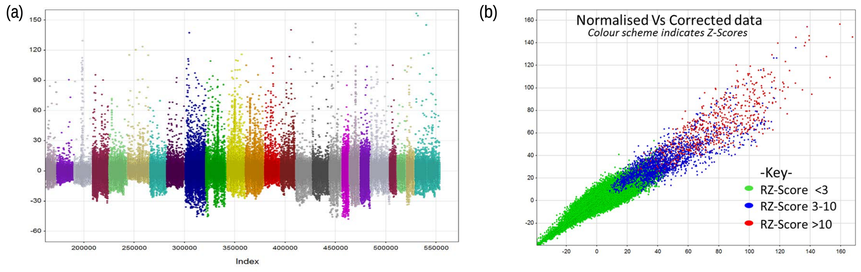


Figure 5. (a) Distribution of compound activity across the HTS with each screening run represented by a different colour (b) Correlation between percentage control data on X axis and corrected percentage control data using Genedata algorithm for plate effects and patterns. Colour scheme indicates RZ-score as shown in the figure legend.

Approximately 5,300 primary hit compounds were identified during the primary screen based on RZ-score >3.6 from normalized and corrected data.
Following duplicate re-testing at the hit confirmation stage approximately 500 compounds were selected for potency determination as duplicate 10-point concentration response curves in primary Nano-BiT® and additional cell toxicity (CTG) assays. The curve fit analysis was performed using Activity Base software and the correlation between replicate values is shown in Figure 6

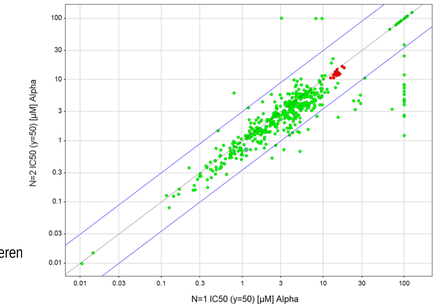


Figure 6. Potency determination with 10-point concentration response curves. The compound activity is shown as IC_{50} values and correlation between duplicate runs (referen compound is shown in red)

7 SUMMARY AND CONCLUSIONS

- A low throughput assay protocol developed by our Partner was successfully converted into a format amenable to HTS
- Overcame significant scientific and technical challenges to successfully screen 0.5M compounds with robust assay performance
- Post potency determination, ~250 potent compounds identified with structural diversity and minimal toxicity
- These results highlight Charles River's capabilities to execute challenging cellular HTS campaigns.