Automated Identification of Cardiac Hypertrophy Modulators Using a hipsc-derived Disease Model

Effective drug discovery and development rely heavily on the availability of predictive preclinical models. For decades, target-based drug discovery has focused on immortalised cells to identify and optimise inhibitory or activating molecules. Testing on more complex biological systems takes place only during late stages in the drug development pipeline. Bringing the most relevant biology into the pipeline earlier would help to mitigate late-stage failures due to safety or efficacy concerns. However, complex biological systems are rarely available in the scale required for high throughput screening. Recent developments in human induced pluripotent stem cell (hiPSC) technologies hold great promise to overcome these limitations.

hiPSCs retain patient-specific genetic background information, differentiate into functional cell types, and closely mimic human pathophysiology. Ncardia has developed a system for scaled expansion and differentiation of hiPSCs, generating large batches of cells that can be cryopreserved until use. This enables the same batch of hiPSC-derived cells to be used for both hit identification and lead optimisation.

Successful drug efficacy screening and validation studies require not only a physiologically relevant cell model, but also validated high throughput screening (HTS) protocols to test the effects of drug candidates. To achieve this, Ncardia has automated its cell culture processes in a 384-well microplate format, as well as its assay readouts and data handling. Setting up such a complex workflow for highly sensitive and specialised cells is challenging. Optimal labware and workdeck configurations for automated liquid handling platforms are key to a robust and reliable process, requiring each step to be executed as quickly as possible to avoid variation across microplates. Tecan's Fluent Automation Workstation offers the speed and flexibility required to meet such process-specific liquid handling needs.

This article outlines how the Fluent workstation was used to automate cell culture for Ncardia's beating, hiPSC-derived cardiomyocytes. It also describes the use of these cultured cells to screen >3500 small molecules in a chemically-induced hypertrophy disease model, using a validated phenotypic assay (Figure 1).

Automated Cell Culture Optimisation of hiPSC-derived Cardiomyocytes

Fully automated cell culture protocols and assays are vital prerequisites for HTS campaigns. Ncardia chose the Fluent 780 workstation to enhance its drug discovery services. The first objective was to develop an infrastructure for the automated production of up to 42 pre-cultured 384-well microplates per day, with enhanced reproducibility versus manual culture, to enable screening of >3500 small molecules in a single study. Quality improvement was assessed by inter-well and inter-plate variation of the cell density (degree of confluence) and cell functionality (spontaneous beating quantified by a calcium flux assay). Three main scripts were developed for cell culture of hiPSC-derived cardiomyocytes in 384-well microplates:

- Assay plate coating,
- Cell seeding,
- Medium refreshment for a period of 10 days.

A schematic overview of the automated process optimisation can be found in Figure 2.

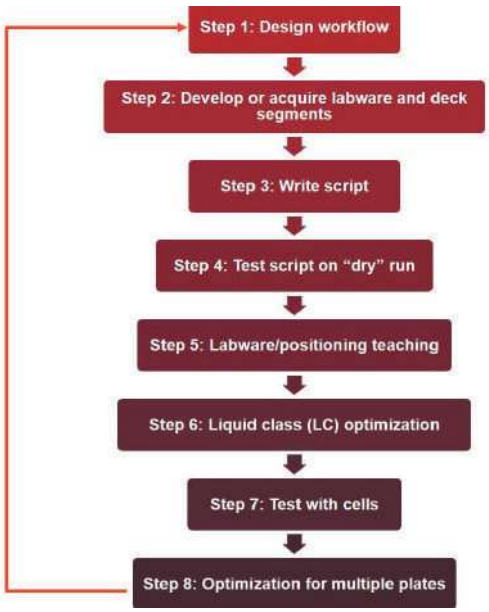

Figure 2: Schematic overview of the optimisation process for automated cell culture of hiPSC-derived cardiomyocytes in 384-well microplates.

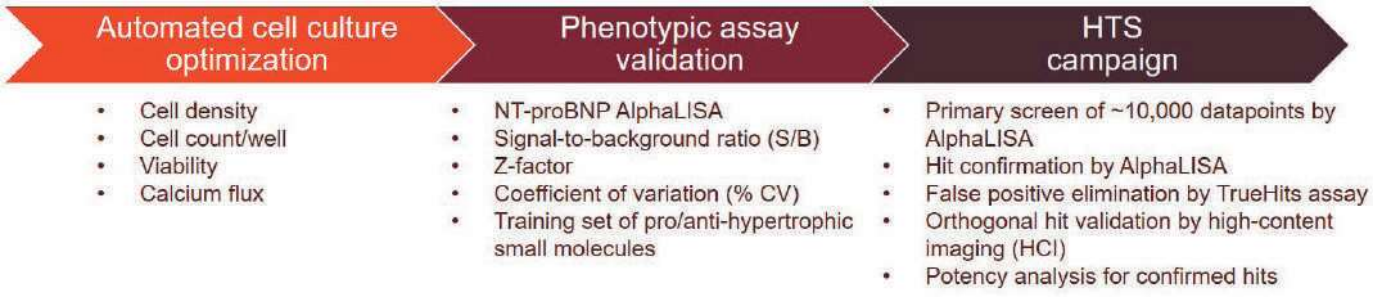

Figure 1: Schematic overview of the automated phenotypic screening workflow using hiPSC-derived cardiomyocytes.

Results

After several rounds of optimisation for labware teaching and liquid class refinement, a high degree of inter-well reproducibility was reached, based on measurements of monolayer confluence per well at 10 days post-seeding, using the Incucyte® Live-Cell Analysis System. The number of cells per well was also quantified using a CyQUANT™ DNA Count Kit and a Spark® 20M multimode plate reader. Before extensive process optimisation, assay plates cultured under fully automated conditions had a higher inter-well coefficient of variation (% CV) compared to assay plates handled manually. However, after process optimisation, assay plates under automated culture had comparable cell numbers with improved inter-well % CV relative to manually cultured plates (Figure 3).

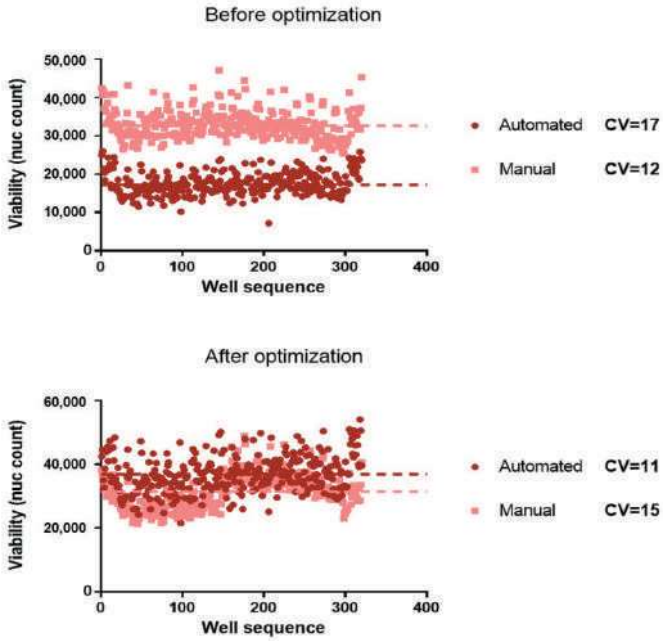

Figure 3: Effect of labware teaching and liquid class optimisation on hiPSC-derived cardiomyocyte cell density, as determined by the CyQUANT viability/nuclear count assay. Two 384-well plates were cultured (one under fully automated conditions and the other manually). After 10 days of culture, the number of nuclei (DNA) per well was measured using the CyQUANT assay. The nuclear count per well, as well as the average nuclear count per plate (dotted line) and inter-well % CV, are shown for manual vs automated cultures, before and after optimisation of automation scripts.

The functionality of the automated hiPSC-derived cardiomyocyte cultures was also assessed at 10 days post-seeding by monitoring the spontaneous calcium flux properties in five assay plates. Figure 4 depicts the baseline measurements for calcium flux in the first and fifth (last) plates according to the handling order. Overall, there were no significant differences in the intra-well, inter-well or inter-plate measurements when comparing critical functional parameters. This data demonstrates that fully automated handling of assay plates for 10 days does not affect the morphology or functionality of hiPSC-derived cardiomyocytes. The automated scripts were therefore suitable for handling multiple assay plates per run in high throughput compound screening applications.

Development of a Hypertrophic Cardiomyopathy Phenotype Assay for HTS in hiPSC-derived Cardiomyocytes

Hypertrophic cardiomyopathy (HCM) is an autosomal dominant disease of the cardiac sarcomere, associated with abnormal thickening of the left ventricular myocardium and resulting in elevated risk for clinical complications, such as progressive heart

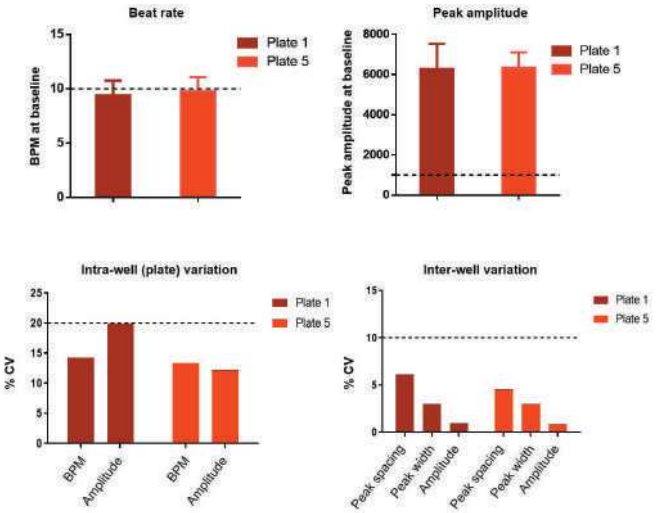

Figure 4: Inter-well and inter-plate variability for assay plates generated using the automated scripts. Five 384-well plates were cultured using fully automated protocols for microplate coating, cell seeding and medium refreshment, and maintained in the automated incubator for 10 days. On day 10, calcium flux in the first and last plates was assessed using the FDSS/µCELL platform. Both plates showed similar beat rate and peak amplitude (top row). Moreover, inter-well and intra-well variation for each plate were within the acceptable ranges, represented by the dashed lines.

failure, arrhythmia and sudden cardiac death[1,2]. The natriuretic peptide fragment, NT-proBNP, becomes more abundant in HCM patients' serum, and is an established clinical biomarker for the diagnosis of hypertrophy. Ncardia has developed a specialised NT-proBNP AlphaLISA assay to detect the secretion of this biomarker in cell culture supernatants from *in vitro* HCM models. This assay was assessed and validated to ensure compatibility with HTS approaches, i.e., to demonstrate high reproducibility and a wide assay window.

Two hiPSC-based HCM models were used in these experiments: a patient-derived hiPSC line carrying an HCM-associated sarcomere mutation in the MYH7 protein, and a WT hiPSC line. Cardiomyocytes were derived from each line at large scale, using stirred tank bioreactors, and were treated with endothelin-1 (ET-1) – a known hypertrophy-inducing peptide typically produced by endothelial and smooth muscle cells. The chemically-induced HCM model in the WT hiPSC line was used for assay development and, later, for primary screening and hit confirmation, whereas the patient-derived model was used for hit confirmation only.

The hypertrophic phenotype of hiPSC-derived cardiomyocytes was assessed by measuring NT-proBNP secretion by AlphaLISA, in the presence of ET-1 and compounds with known anti-hypertrophic abilities. A Spark 20M multimode plate reader was used to detect AlphaLISA signals. The assay performance was evaluated using known quality control criteria, such as signal-to-background ratio (S/B), Z-factor and % CV. This was necessary to ensure the robustness of high throughput compound screening for the novel cell-based platform.

Results

The cardiac hypertrophy assay was robust (S/B >4) and reproducible (Z-factor >0.4 and % CV <20) for both ET-1 induced hypertrophy and inhibition of hypertrophy with the known anti-hypertrophic compound verapamil hydrochloride – used here as a positive control (Figure 5A). Validation of the assay for HTS by testing a larger set of pro- and anti-hypertrophic

compounds in duplicate 384-well plates confirmed the reproducibility of the assay (correlation coefficient >0.9), and its suitability for screening (Figure 5B). This data confirms the successful development of a cardiac hypertrophy assay in hiPSC-derived cardiomyocytes that is appropriate for HTS.

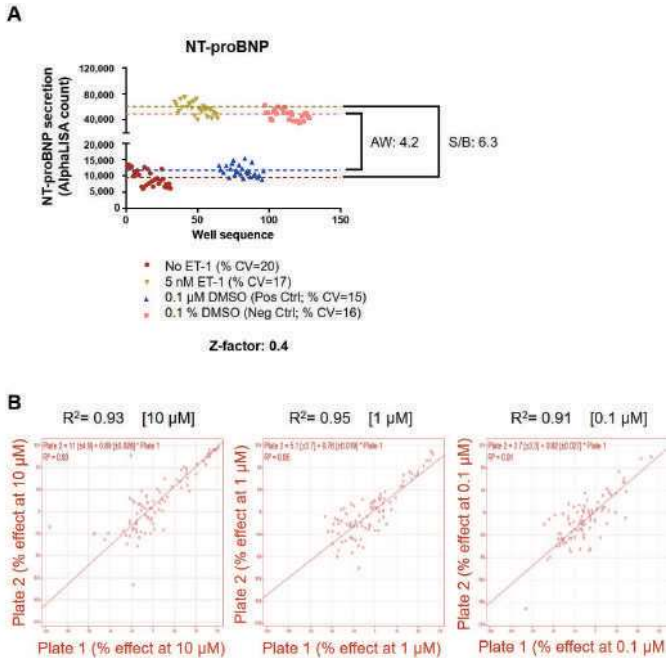

Figure 5: Validation of NT-proBNP AlphaLISA for use with hiPSC-derived cardiomyocytes in HTS. Several replicates of four test conditions were used to assess assay performance in each 384-well plate. All groups showed low variation (% CV <20). (A) The assay was robust, as evidenced by an S/B of 6.3 and an assay window of 4.2. A Z-factor of 0.4 confirmed the reproducibility of the assay for high throughput compound screening. (B) For further validation, a selected panel of 96 anti- and pro-hypertrophic compounds at three concentrations (0.1, 1 and 10 µM) was screened in duplicate. The duplicate plates showed high reproducibility at all concentrations tested (correlation coefficient >0.9).

High Throughput Efficacy Screening for Identification of Anti-hypertrophy Drugs with Lead-like Properties

After successful development of the ET-1 induced HCM model, and validation of the clinically relevant NT-proBNP AlphaLISA for HTS, a primary high throughput compound screen was performed using three chemical libraries:

- the Prestwick library, consisting of 1180 FDA-approved, highly chemically and pharmacologically diverse small molecules with a high level of hit-like properties (screened at 0.1 and 1 µM),
- a library of 450 FDA-approved compounds (screened at 1 µM),
- a phenotypic diversity library of 2200 compounds consisting of diverse target classes and signallling pathway modulators (screened at 1 µM).

The entire screen consisted of 3830 compounds (5010 data points, as the Prestwick library was screened at two concentrations per compound) in fifteen 384-well microplates. Each plate consisted of 24 replicates of 0.1 % DMSO plus ET-1 (MIN effect) and 0.1 µM verapamil hydrochloride plus ET-1 (MAX effect), enabling assessment of the quality of each plate throughout the screening.

Results

As shown in Figure 6A, all the plates had a high S/B (>4) and robust Z-factor (>0.4), except for one plate which had a Z-factor very close to the lowest accepted limit (0.32). This data shows that all plates met the predefined quality criteria and did not require rescreening. As a next step, the percent inhibition (PIN) of all compounds was calculated relative to the MAX effect (100 % inhibition). Based on distribution analysis of the compound effect at different cut-offs, the threshold for considering a compound as a hit was set at PIN >40 %. According to this predefined cut-off, 341 compounds were identified as primary hits (Figure 6B).

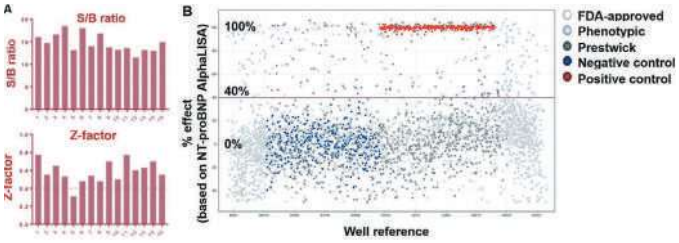

Figure 6: Reproducibility and robustness assessment for primary screen. (A, Top left) S/B (MAX signal vs MIN signal) was assessed for 15 screening plates, with all 15 plates displaying an S/B >10. (A, Bottom left) 14 plates had Z-factor >0.4, and one plate had Z-factor of 0.32. (B) Each dot represents the effect of a single compound at 1 µM presented as percentage inhibition relative to MAX effect (100 % inhibition, red dots). The three libraries screened are shown in different colours. Blue dots depict MIN effect. Compounds with PIN >40 % (above red line) were considered as primary hits.

Following the primary screen, a hit confirmation analysis was performed for 341 compounds, using three distinct assays:

- NT-proBNP secretion AlphaLISA (technical duplicate),
- the AlphaScreen® TruHits™ assay to deselect false positives,
- high content imaging (HCI) to assess intracellular proBNP expression.

Based on the data obtained from the NT-proBNP AlphaLISA, 248 compounds showed a PIN >40 % (Figure 7A). To deselect false positive compounds (i.e., compounds that interfere with one of the components of the AlphaLISA technology, reducing the signal intensity), an AlphaLISA TruHits assay was performed. Two versions of the assay were used to exclude compounds interfering with either biotin (biotin mimics) or signal intensity (quenchers or scatterers) (Figure 7B). Based on the results, 192 of the hits with PIN >40 % in the NT-proBNP AlphaLISA had TruHits activity <20 % in both versions of the TruHits assay. This resulted in a final hit identification rate of 5%, which is a reasonable rate for a phenotypic cell-based screening using FDA-approved libraries.

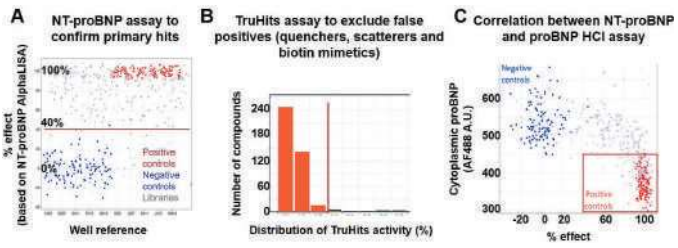

Figure 7: Hit confirmation with three distinct assays. (A) Primary hits were first confirmed by repeating the NT-proBNP AlphaLISA at a 1 µM concentration for each compound. Compounds with PIN >40 % (red line) were confirmed as hits. (B) TruHits assays were performed to deselect false positives. The graph indicates compounds identified as colour quenchers, light scatterers (insoluble compounds), singlet oxygen quenchers and biotin mimetics (right of red line). Compounds with PIN >40 % in the NT-proBNP AlphaLISA, and TruHits activity <20 % (left of red line) were selected as confirmed hits. (C) The NT-proBNP AlphaLISA was multiplexed with HCI to confirm intracellular proBNP expression. The graph depicts the PIN effect of compounds in the NT-proBNP AlphaLISA (X axis) vs. the intracellular proBNP expression (arbitrary fluorescence units, AU) in proBNP+ cells (Y axis). Red dots represent the MAX effect reduction in proBNP levels, and blue dots represent the MIN effect.

Additionally, the NT-proBNP AlphaLISA was multiplexed with HCI of proBNP and DAPI (nuclear DNA stain) to quantify intracellular proBNP expression (orthogonal assay) and evaluate any cytotoxic compound effects, respectively. As depicted in Figure 8C, the assay positive control (5 nM ET-1 + 0.1 µM verapamil) reduced intracellular proBNP expression levels compared to the negative control (5 nM ET-1 + 0.1 % DMSO). Furthermore, some hits reduced the proBNP expression in both assays (NT-proBNP AlphaLISA and HCI intracellular proBNP), while others only showed inhibitory effects in the NT-proBNP AlphaLISA.

Finally, a potency analysis for the 192 confirmed hits was performed, using the NT-proBNP AlphaLISA. Dose-response curves were set up with eight concentrations per compound, ranging from 1 nM to 5 µM. The potency analysis was set up within eight plates that passed the predefined quality criteria (data not shown). This analysis revealed that hits had varying potencies, as shown in Figure 8. Some drugs showed low potency (estimated EC50 >10 µM), while others showed moderate potency (estimated EC50 <1 µM). Most compounds in the latter group had well-fitted 'S curves', enabling a precise calculation of EC50 values. Many confirmed hits had lead-like properties (sub-µM potency, favourable DMPK properties, good safety profiles, etc.) and are currently being further investigated as potential HCM therapeutics.

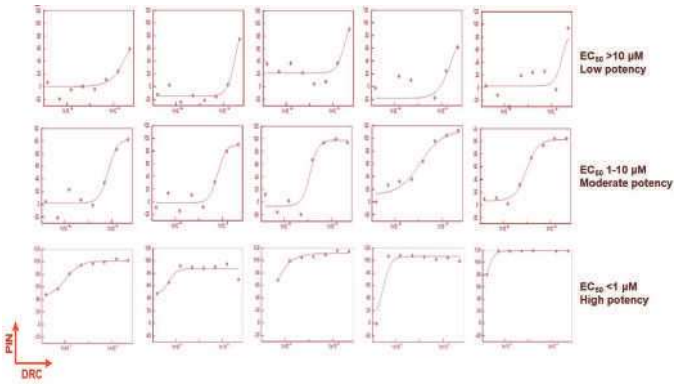

Figure 8: Potency analysis for the 192 confirmed hits. 8-concentration dose-response curves, ranging from 1 nM to 5 µM, were tested for each compound, identifying varying potencies. The top row depicts representative compounds with low potency (EC50 estimated at >10 µM). The middle row shows representative compounds with moderate potency (calculated EC50 from 1–10 µM). The bottom row depicts representative compounds with high potency (EC50 estimated at <1 µM).

Summary and Conclusions

This study demonstrates how hiPSC-derived cells provide a physiologically relevant disease model that can be used in a high throughput screening campaign for assessment of drug efficacy. Several hurdles were overcome to enable this hypertrophic cardiomyopathy phenotypic screen. First, Ncardia's industry-leading, scalable hiPSC-derived cell manufacturing enabled generation of sufficient quantities of cells from the same batch to execute a complete high throughput phenotypic screen, all the way from assay development to hit validation. The Fluent workstation allowed these hiPSC-derived cardiomyocytes to be cultured for a prolonged time period (≤14 days) in a fully automated way, with high well-to-well and plate-to-plate reproducibility.

The phenotypic assay described was robust and reproducible (S/B >4, Z-factor >0.4 and % CV <20), allowing 42 384-well microplates to be handled per run. This offers the speed and flexibility needed for the validation and execution of any phenotypic screening campaign using specialised

hiPSC-derivatives. As summarised in Figure 9, the phenotypic screen of >10,000 data points resulted in 341 initial hits. Hit confirmation assays narrowed this number down to 192 final hits. EC50 values were obtained for all 192 confirmed hits, and many of these have lead-like properties. Most importantly, these results hold great promise for the development of safer and more effective drugs for hypertrophic cardiomyopathy patients, and facilitate Ncardia's mission to help get better drugs to patients faster.

Figure 9: Schematic summary of the phenotypic HTS campaign performed by Ncardia on the Fluent Automation Workstation to identify anti-hypertrophic compounds using hiPSC-derived cardiomyocytes (CRC = concentration-response curve).

REFERENCES

1. Maron, BJ et al. Images in cardiovascular medicine. Extreme left ventricular hypertrophy. Circulation, 1995, 92(9), 2748.
2. Kuwahara, K et al. The effects of the selective ROCK inhibitor, Y27632, on ET-1-induced hypertrophic response in neonatal rat cardiac myocytes – possible involvement of Rho/ROCK pathway in cardiac muscle cell hypertrophy. FEBS Letters, 1999, 452(3), 314-8.

Elena Matsa

Elena is the Director of Discovery Technology at Ncardia. She obtained her PhD in stem cell biology in 2010, and subsequently worked as a post-doctoral researcher at the University of Nottingham, and the Stanford University School of Medicine. She has extensive experience and high impact publications in modeling of human cardiac disease in iPSC-derived cardiomyocytes.

Email: elena.matsa@ncardia.com

Lucía Bruzzone

Dr. Lucía Bruzzone is an application specialist at Tecan Switzerland. She studied molecular biology and biotechnology at the University of Buenos Aires in Argentina. During her PhD, she focused on cell biology and genetics at the Institut Jacques Monod in Paris, France. She joined Tecan in 2019 and focuses on the development and support of applications on Tecan's liquid handling platforms in the cellomics and proteomics area.

Email: lucia.bruzzone@tecan.com