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Technical Brief

High-throughput differential scanning fluorimetry (DSF) and cellular thermal shift assays (CETSA): Shifting from manual to automated screening

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ABSTRACT

Biophysical affinity screening is increasingly being adopted as a high-throughput hit finding technique in drug discovery. Automation is highly beneficial to high-throughput screening (HTS) since a large number of compounds need to be reproducibly tested against a biological target. Herein, we describe how we have automated two biophysical affinity screening methods that rely on a thermal shift in protein melting temperature upon small molecule binding: differential scanning fluorimetry (DSF) and the cellular thermal shift assay (CETSA).

1. Introduction

Generating new small molecule leads through high-throughput screening (HTS) is a key step in drug discovery [1]. Traditionally, HTS assays evaluate the biological activity of a target, assessing a functional readout using a biochemical or cellular assay. More recently, biophysical affinity based methods have been increasingly adopted as the portfolio shifts towards targets that lack measurable functional activity [2,3]. These assays measure the direct binding of a small molecule to a biological target. High-throughput affinity screening techniques include affinity-selection mass spectrometry (AS-MS) [4], DNA-encoded libraries (DEL) [5], differential scanning fluorimetry (DSF) [6], and the cellular thermal shift assay (CETSA) [7].

The AstraZeneca (AZ) HTS department has been working to automate two thermal shift assays at scale: DSF and a HiBiT-based CETSA (Promega, Madison, WI). Thermal shift assays assess ligand-induced conformational stabilization of proteins as they undergo a temperature induced transition from a native folded (usually soluble) to a denatured unfolded (usually insoluble) state. This is measured by a shift in the melting temperature of a protein (Tm), which is defined as the temperature at which half the population is unfolded/denatured. Dye-based DSF monitors this process in an isolated protein system using a fluorescent hydrophobic dye, e.g., SYPRO orange [8]. As a protein unfolds it exposes hydrophobic residues that cause an increase in dye fluorescence. The Tm is measured using a real-time PCR instrument; if a compound binds to the protein, the protein’s Tm usually shifts to a higher temperature. CETSA allows measurement of a compound binding to an intracellular target by quantifying the amount of soluble protein remaining in a cell following a heat shock (usually at the Tm of the protein of interest), since compound binding usually increases the amount of soluble, folded protein. There are several different readouts available for CETSA, such as western blot, mass spectrometry, AlphaLISA and split-reporter systems such as HiBiT [9]. The use of HiBiT tagging allows the level of soluble protein to be quantified using luminescence with a number of advantages for cost-effective application at scale in high-throughput screening [10,11].

The AZ HTS department deploys HighRes Biosolutions’ (Beverly, MA) modular platforms for automated compound screening [12]. These platforms utilize Flex Carts that have been designed to cover a key series of tasks in an assay process, e.g., dispense carts, reader carts, imaging carts, incubator carts. Flex Carts can therefore be selected for the assay in question, allowing “plug and play” operation. The carts connect to MicroDocks that provide them with power, communication, gas and air; they also define the automation system layout. Two Flex Carts can be docked together to form a semi-automated bay and up to five Flex Carts can be docked around a central KUKA LBR iiwa robot and fixed table on a CoLAB MicroStar platform. Novel Flex Carts can also be developed according to assay needs: for example a quantitative polymerase chain reaction (qPCR) cart containing three QuantStudio 7 (qPCR) System 7 Pro Real-Time PCR Systems (Thermo Fisher, Waltham, MA) was recently integrated. This technical brief will discuss the challenges and impact of establishing high-throughput automated DSF and HiBiT CETSA assays.

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2. Materials and Methods

2.1. Automated DSF

Compounds in 100% DMSO are acoustically dispensed into PCR plates (Roche LightCycler PCR plates 04729749001) using the Echo 650 (Beckman, Brea, CA), with 32 wells of neutral control (100% DMSO) per plate. The final concentration for single point screening is 100 µM (50 nL of 10 mM stock acoustically dispensed into PCR plates). Thermally sealed assay ready plates containing compound at the desired concentration are loaded into the SteriStore automated microplate incubator (HighRes Biosolutions) set to 4 °C and ambient humidity. Plates are removed sequentially and are de-sealed using the X-Peel automated plate seal remover (Brooks Life Sciences, Burlington, MA) before being loaded into the Echo 650 where 5 nL of the SYPRO orange dye is dispensed into each well at a predefined concentration (this should be selected to maximize the signal intensity whilst using the lowest amount of dye possible, in this 5000x stock solution in 100% DMSO). The plates are then transferred to the Certus Flex (Gyger Fluidics GmbH, Bern, Switzerland) or Multidrop Combi (Thermo Fisher) liquid dispenser where 5 µL of the protein (at a pre-defined concentration and buffer composition dependent on the protein) is dispensed to every well. A screen of 100,000 compounds requires ~800 mL of protein at the final concentration. In this case, the final protein concentration 2.5 µM, meaning the entire screen consumed 49 mg of protein. The plate is then sealed with LightCycler 480 Sealing Foil using a PlateLoc Thermal Microplate Sealer and centrifuged in the MicroSpin (HighRes Biosolutions) for a quick pulse spin at 100 g. Plates are returned to the SteriStore and incubated at 4 °C for at least 30 min. After 30 min, the first plate is removed and loaded onto the Q57 Quantstudio 7 Flex Real-Time PCR System (Thermo Fisher). Fluorescence is read over a temperature gradient and ramp rate defined for each target protein (e.g., heating from 25 to 80 °C at 1 °C per minute). The plate is then unloaded from the Q57 and disposed of into a plate waste bin. Plates are read sequentially until all plates have been read. The protocol is designed and scheduled using HighRes Biosolutions Cellario scheduling software (Supplementary Figure 1A). Data is analyzed using Genedata Screener and the Tm determined using the maximum of the 1st derivative. A significant shift in Tm is defined as ΔTm ≥3 SD of the neutral (DMSO) control across all plates in the screening campaign.

2.2. Automated CETSA

Compounds in 100% DMSO are acoustically dispensed into assay ready PCR plates (BioRad PCR plate HSP3805) using the Echo 650. The final concentration for single point screening is 50 µM (100 nL of 10 mM stock acoustically dispensed into PCR plates). Thermally sealed assay ready plates containing compound at the desired concentration are loaded into the SteriStore set to ambient conditions (22 °C, 0% CO2, 65% humidity). Plates are sequentially unloaded, de-sealed using the X-Peel and transferred to the Multidrop Combi liquid dispenser where 10 µL cells in HBSS (Hank’s balanced salt solution, Gibco, Thermo Fisher) (2500 cells/well) (adherent cell line with HiBiT tagged protein of interest) are dispensed into every well. The cells are then loaded onto the VPrep pipetting station (Agilent) and 10 µL is transferred (being careful not to disturb the pellet) into a 384 low volume plate (Greiner 784075s) that have been loaded into the NanoServe Labware Stacker Carousel (HighRes Biosolutions). The BioRad plate is returned to the SteriStore whilst the Greiner plate is loaded into the PheraSTAR FSX (BMG, Ortenberg, Germany) for a luminescence measurement. Following the read step, the plate is returned back to the NanoServe. The protocol is designed using HighRes Biosolutions Cellario scheduling software (Supplementary Figure 1B). Data is analyzed using Genedata Screener, and hits are defined using a suitable z-score cut-off (in this case –9).

3. Results and Discussion

3.1. Automating DSF

Originally the DSF assay was run as a manual screening process shown in Fig. 1A, starting with assay ready PCR plates containing compounds. SYPRO Orange dye and the target protein are dispensed into plates using the Echo and Certus dispensers respectively, followed by a brief centrifuge step. Sealed plates are stored at 4 °C (for a minimum of 30 min), before being loaded into the Q57 system for the heat/read step that takes ~50 min/384-well plate (e.g., heating from 25 to 80 at 1 °C per minute). For a DSF assay, 100 K compounds are routinely tested, screening ~350 assay plates in total; running this assay manually is time consuming and restrictive as assay plates need to be manually loaded into the Q57Pro machine every 50 min throughout the day.

System setup: Automated DSF was enabled by linking a SteriStore, Echo and Q57 Flex Carts (Fig. 1B) on a Semi-Automated-Bay automation platform. The SteriStore is an incubator; for DSF it is set to 4 °C. The Echo cart contains an Echo 650 dispenser, a Multidrop Combi dispenser, a X-Peel seal-remover, a Plate-Loc plate-sealer and a MicroSpin centrifuge. The Q57 cart contains three Quantstudio 7 Pro Real-Time PCR System machines. This is because the Q57 step is the slowest step in the automated process, taking around 60 min per plate. By having three Q57 machines the heat/read step can process 3 plates in 60 min, speeding up the process by 2/3rds. The Echo cart was placed in the master MicroDock location on the Semi-Automated-Bay platform, the Q57 cart in the slave MicroDock location, and the SteriStore Incubator was positioned adjacent to the system (Fig. 1C). Since the SteriStore was not docked to a MicroDock it was plugged into the house services.

Certs vs. Multidrop: When DSF is run manually, a Certus is usually used for the protein dispense. Due to resource availability, the Echo cart was originally set up with a Multidrop Combi. Protein dispensing by a Multidrop was therefore tested, and Multidrop dispensing gave comparable results to a Certus Flex for the target in question (Tm = 48.75, 3 SD = 0.41 for Certus, Tm = 49.2, 3 SD = 0.43 for Multidrop) allowing the assay to be fully automated (Fig. 1D). For other targets (data not shown) the Certus gave more reproducible results with lower% coefficient of variation and so a Certus dispenser is currently being installed on the Echo cart.

Reagent dispensing: Initially, the automated assay was set up to allow for “just in time” dispensing. Each plate was dispensed just before being passed to the Q57. However, this meant that the multidrop would sit idle for the length of time the Q57 took to process a plate before the next plate was dispensed (roughly 50 min). To prevent tube clogging, the Multidrop was primed and purged to waste before dispensing to each plate, wasting valuable protein reagent. Therefore the protocol was amended to process all dispense steps and set the assay up as quickly as possible, with plates being dispensed sequentially at the start of the run. The plates were then stored at 4 °C in the SteriStore before being heated/read as it was shown that the assay was stable for up to 10 h (Fig. 1E). This meant there was no need to prime and purge on every plate, reducing the total volume of protein solution required by 10%. It must be noted that the “stability” of the assay is protein dependent and so the 60 second centrifugation at 400 g using the MicroSpin, plates are loaded onto the VPrep pipetting station (Agilent) and 10 µL is transferred (being careful not to disturb the pellet) into a 384 low volume plate (Greiner 784075s) that have been loaded into the NanoServe Labware Stacker Carousel (HighRes Biosolutions). The BioRad plate is returned to the SteriStore whilst the Greiner plate is loaded into the PheraSTAR FSX (BMG, Ortenberg, Germany) for a luminescence measurement. Following the read step, the plate is returned back to the NanoServe. The protocol is designed using HighRes Biosolutions Cellario scheduling software (Supplementary Figure 1B). Data is analyzed using Genedata Screener, and hits are defined using a suitable z-score cut-off (in this case –9).
stability must be tested for every protein target.

**Output:** A fully automated DSF screening campaign was performed using the Semi-Automated-Bay platform (Fig. 1C). 100 K compounds were screened and following hit triage, 4 compounds were confirmed as binding to the target via 2D nuclear magnetic resonance (NMR). The automated process facilitated out-of-hours screening, increasing daily plate throughput and reducing project screening timelines by 40%.

### 3.2. Automating CETSA

The Nano-Glo HiBiT Lytic Detection System [13] works by adding an 11 amino acid HiBiT peptide tag to a protein of interest expressed in a cell line. These cells are dispensed into an assay ready plate containing compounds to be screened. The plate is then heated to an optimized temperature causing cellular protein unfolding. Following this, Nano-Glo HiBiT Lytic Detection Reagent (Promega) is added that lyses the cells and introduces LgBiT, a larger subunit that binds to HiBiT with high affinity to form the luminescent NanoBiT enzyme. If the HiBiT tagged protein remains folded due to compound binding, the LgBiT binds to the HiBiT to reconstitute the luminescent NanoBiT enzyme. The cells and lytic detection reagent need to be mixed thoroughly and the mixture is then centrifuged to pellet the unfolded protein. The supernatant contains all soluble, folded protein due to compound binding and is transferred to a new assay plate ready for a luminescence read in a plate reader. The luminescence signal is directly proportional to the amount of HiBiT tagged protein; a luminescent signal therefore indicates compound binding to the target protein since this causes thermal stabilization of the target, increasing the amount of folded protein present in the soluble fraction.

**Heating step:** Initially, QS7 Pro qPCR instruments on the QS7 Flex Cart were used to heat plates for CETSA experiments on the HighRes CoLAB platforms. However, QS7 Pros are also required for automated DSF screening, creating a resource clash. Moreover, there is no requirement for a fluorescent read during heating for CETSA. Therefore, the INHECO On Deck Thermal Cycler (ODTC) (Fig. 2 Ai) was deployed on the HTS CoLAB platforms. The ODTC accurately and uniformly heats 384-well PCR plates and is cheaper and has a significantly smaller footprint than the QS7 Pro. The ODTC and the ODTC processor were installed on the CoLAB fixed table (Fig. 2 Aii, iii, iv), leaving space for another Flex Cart to be docked if required on the CoLAB system.

**Mixing step:** HiBiT CETSA assays can require a mix, spin and transfer step post lysis to separate folded and unfolded protein [14]. The mix step, which can be critical for obtaining high quality assay performance on some targets (Fig. 2B), was optimized using a fixed tip system (V-Prep/Bravo, Agilent, 10×10 µL mix). However, automating the entire process necessitated access to two docked fixed-tip pipettor stations, severely limiting the potential screening throughput (Fig. 2C). Implementation of a BioShake 500 elm (QInstruments) on a Dispenser Flex cart has enabled effective mixing, removing the need for an additional pipette station and obtaining S:B and Z' statistics comparable to the

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**Fig. 1.** Automating Differential Scanning Fluorimetry (DSF) assays. A) Manual DSF assay process. SYPRO orange dye is acoustically dispensed into assay ready PCR plates using the Echo 650 (Beckman). Protein is then dispensed using the Certus Flex (Gyger), followed by a centrifugation step. Plates are then sealed and incubated at 4 °C for at least 30 min before being read in a Q57 QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher). B) Diagram of the Echo Flex cart, SteriStore & Q57 Flex cart. C) Automated DSF setup on a HighRes Semi-Automated Bay platform: the Echo Flex cart in the master MicroDock location, the Q57 Flex cart in the slave MicroDock location and the SteriStore Incubator positioned adjacent to the system, plugged into house services. D) DSF melt curves across 48 wells in the Roche plates comparing Certus Flex (Gyger) vs. Multidrop (Thermo Fisher) dispensing of protein. Both methods result in very similar Tm and standard deviation (SD) values. E) The stability of the assay was tested over 10 h. Reagents were prepared at 0 h and stored at room temperature, as they would be during a screening run. Every hour, protein (10 µL of 2.5 µM) was dispensed into 6 wells of a 384-well plate and the Tm of the protein was measured. The Tm did not shift over the course of 10 h (the maximum length of a screening run), and the 3*RSD across the 10 hour period was <1 °C.

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Automated dispenser cleaning: At the end of the automated assay protocol, both Certus and Multidrop liquid dispensers must be cleaned to prevent clogging of the tubing that could make them unusable for future runs. Multiport VICI rotary valves (VICI Valco Instruments, Houston, USA) were incorporated into the automated screening protocol to permit just-in-time automatic liquid dispenser cleaning at the end of a screen. These multi position micro electric valve actuators enable automatic dispenser cleaning by permitting switching between assay reagents and wash solutions (e.g., water and 70% ethanol) at the end of a run. Once reagent dispensing is complete, wash solutions can automatically flow through the dispenser, cleaning the dispenser valves/tips and associated tubing.

Cellario scripting: Since CETSA can enable screening against challenging targets, there are often no control compounds available for a HTS. In place of this, unheated plates can be used as an artificial positive control. A Cellario software script (Supplementary Fig. 1B) was developed that allows users to skip the heating step for selected plates in the screening run, enabling unheated plates to be placed intermittently throughout the run. This enables the user to monitor the assay statistics throughout the entirety of the screening process.

Output: CETSA enables identification of small molecules that engage with the target protein in the native cellular environment and offers advantages for proteins that have no measurable functional activity or that are difficult to express and purify at the scale required for high-throughput screening, often due to stability issues or the presence of regions of intrinsic disorder. The HiBiT CETSA protocol can be applied to multiple targets, and by automating this process, up to 0.5 M compounds can be screened in single-point format in < 4 weeks.

4. Conclusion

As the drug discovery portfolio shifts towards more intractable targets, where there is often no known binding site, tool compound or measurable functional activity, the demand for affinity based screening technologies has increased. Affinity screening enables identification of small molecules that bind to the target protein without measuring a functional endpoint or requiring prior structural knowledge. Thermal shift assays measure ligand binding induced thermal stabilization of a protein; DSF and CETSA are two thermal shift assays that measure ligand binding in isolated protein and cellular systems, respectively. Whilst both are amenable to high-throughput screening, these assays are time consuming and complicated to run manually, and the absence of automation means that screening is limited to within working hours. Until now, automated DSF and CETSA screening has not been possible at AZ. Successful implementation of automation of both technologies has transformed our affinity screening capabilities, with screening times reduced by >33% and collections of >500k compounds screened successfully. We are now able to run automated high-throughput thermal shift assays for both isolated protein and cellular assays, and these systems have become a key part of our core screening capabilities.
Declaration of Competing Interest

All authors are employees of AstraZeneca. The authors declare that they have no conflict of interests.

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Supplementary materials

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