EMPLOYING CETSA IN PRIMARY SCREENING FOR P53 BINDERS

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INTRODUCTION

Drug targets such as p53 can be considered challenging or 'undruggable' for multiple reasons such as lack of tools, assays, lack of information about enzyme activity or cellular localization.

Using our patented Cellular Thermal Shift Assay - CETSA®, we screened for p53 binders in intact SK-BR-3 breast cancer cells using commercial libraries. Hit follow-up yielded hit-matter and orthogonal activity data confirmed the effect on p53 activity. This approach enables rapid hit generation by prioritizing compounds active in live cells, saving time and cost compared to traditional HTS, which relies on recombinant proteins or engineered systems that may not reflect true cellular biology.

PRINCIPLE OF SCREENING USING CETSA

CETSA for high-throughput screening uses AlphaLISA® to detect compound-induced changes in protein stability by measuring soluble, non-aggregated protein levels after heating. This assay format enables rapid, miniaturized screening directly in cells or lysates (Figure 1). In early drug discovery, CETSA offers valuable insight by identifying direct binders without modifying the protein or compound. It streamlines hit identification by focusing on compounds active in native cellular environments, improving relevance and reducing time spent on false positives from traditional biochemical assays.

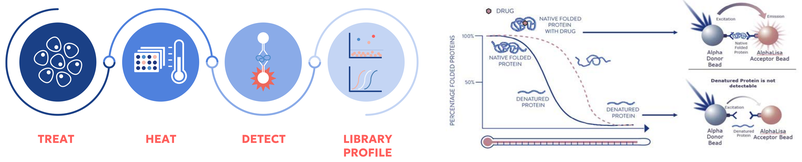

Figure 1. CETSA Screening workflow using AlphaLISA

ASSAY DEVELOPMENT & PILOT SCREENING

A CETSA protocol was established in SK-BR-3 breast cancer cells with p53 (R175H) gain-of-function mutation (Figure 2). Assay robustness was ensured by optimizing statistics and calibrating screening concentration. A single-point pilot screen was performed at 30 µM using the Bioactives compound library (Figure 3).

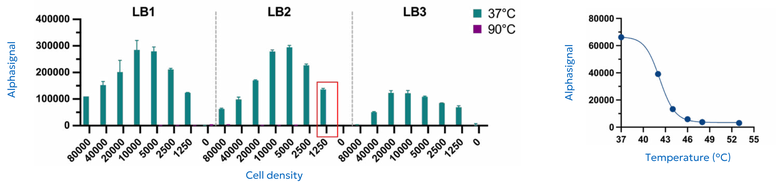

Figure 2. Analyte titration & Lysis Buffer (LB) selection and Melt Curve profiling

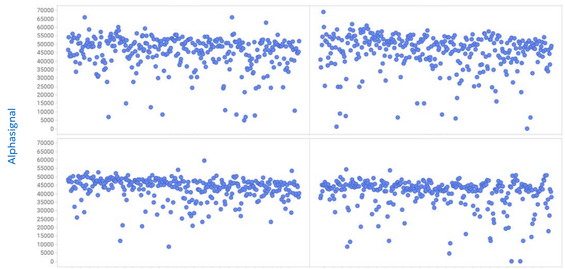

Figure 3. Pilot Screen of Bioactives library

PRIMARY SCREENING

The 16K high throughput screen was completed with good assay statistics and stable signal generated across all plates. Assay controls provided good Z' values. The identified hits were from diverse plate positions and no edge or systematic effects were observed. Data QC methodologies are automated in our data analysis pipeline to ensure a consistent approach to control well removal if needed.

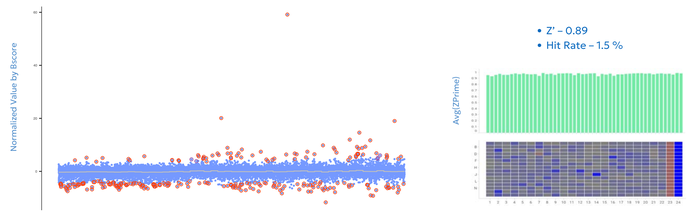

Figure 4. Scatter plot visualising the results of the p53 screening campaign, Z´factor and hit distribution.

HIT RECONFIRMATION

Hit reconfirmation studies were done with duplicated 5-point concentration response (CR) curves. Twelve compounds were confirmed as hits in SK-BR-3 cells and 11 also yielded concentration dependent stabilization in MCF7 cells. Only 7 out of 12 compounds produced a CR curve in SK-BR-3 lysate. Figure 5 illustrate results for three example compounds.

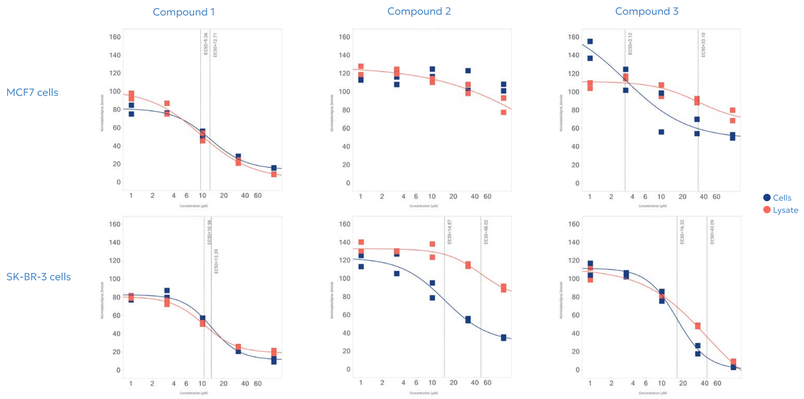

Figure 5. Examples CR curves for three of the hit compounds.

DOWNSTREAM FUNCTIONAL ASSAYS

Quantitative real-time PCR (qPCR) assay for two known target genes of p53 were used to evaluate the parmacological relevance of the 12 hit compounds (Table 1). All compounds that gave a response on PMAIP1 also have an effect on CDKN1A. Increased expression of these genes are indictive of restored p53 function, but they do not report on potential mutant p53R175H gain of function effects.

Gene	Upregualted	Unchanged	downregulated
CDKN1A (p21WAF1)	8	4	1
PMAIP1 (NOXA)	5	7	0

Table 1. The effect of hit compounds on p53 target genes CDKN1A and PMAIP1 in SK-BR-3 cells after 3 h assessed by qPCR. P>0.001 by Student's T-test

PROTEOME WIDE CETSA PROFILING OF HITS

Lysate prepared from SK-BR-3 cells was incubated with 30 µM of each compound and then subjected to a CETSA protocol followed by MS detection (DIA, Orbitrap(TM) Astral(TM) Mass Spectrometer) in order to assess the proteome wide effects of each compound. Compound 1 show many protein hits associated with redox functions, whereas compound 2 has few hits. Noteably, one of the top stabilized hits with compound 2 was FAM193A, a protein that has recently been described as a positive regulator of p53 activity (Szwarc et al 2023).

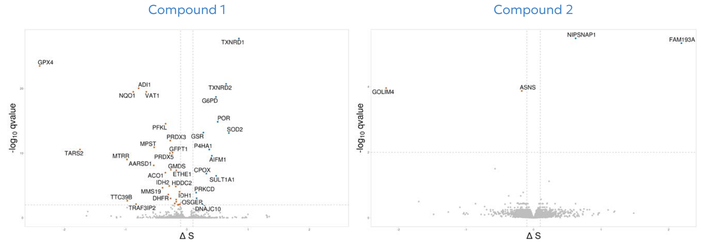

Figure 6. Examples of proteome wide thermal stability profiling of two of the hit compounds.

SCREENING PIPELINE

The HTS pipeline automates the core CETSA protocol allowing precise control of time and temperature sensitive steps. The system is designed around a Biomek i7 series liquid handling platform and comprises dispensation, incubation, heating and liquid handling steps. Time-flow critical steps are precisely controlled allowing for full HTS runs to be autonomously scheduled.

Pelago Bioscience's high throughput platform

CONCLUSION

We used CETSA to identify p53 binders in breast cancer cells, revealing compounds that stabilize p53 activity and offer potential for cancer therapy.

KEY BENEFITS

- Rapid assay development
- Works with all native (unmodified) cells
- Scalable library selection 1k-100k+
- Unparalleled QC and robustness
- Complete automation of time critical workflows

If you're interested in our p53 study, our approach, or using CETSA HT for your own targets, feel free to contact us via email: **contact@pelagobio.com**

References

1. Rowlands H. et al. High throughput screening of 0.5 Million compounds against CRAF using Alpha CETSA®. SLAS Discovery 1:S2472-5552(23)00012-6 (2023)
2. Henderson MJ. et al. High-Throughput Cellular Thermal Shift Assays in Research and Drug Discovery. SLAS Discovery. 25(2):137-147 (2019)